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THE PREDICTION OF ENERGY BALANCE OF DAIRY COWS FROM ANIMAL, FEED, AND MILK TRAITS WITH SPECIAL REGARD TO MILK FATTY ACIDS

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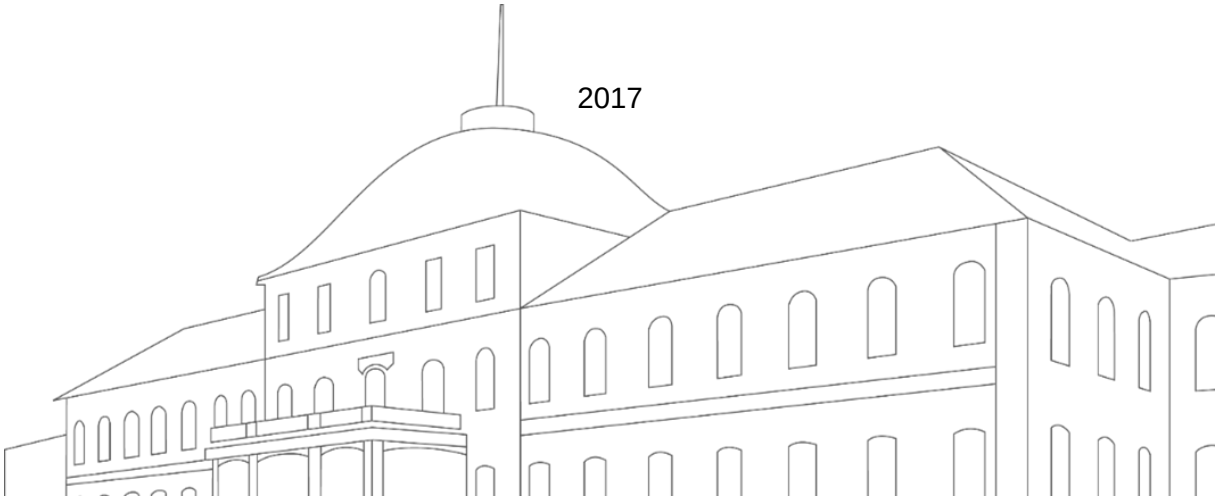

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For

My Mother

My Grandma

My Sister

“Essentially, all models are wrong, but some are useful.”
~ George E. P. Box

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ABBREVIATIONS

%CV	Percent coefficient of determination
ACP	Acyl carrier protein
AdaLasso	Adaptive least absolute shrinkage and selection operator
ADANET	Adaptive elastic net
AICC	Corrected Akaike Information criterion
ALA	α-Linolenic acid (C18:3c9,c12,c15)
BCFA	Branched-chain fatty acid
BCS	Body condition score
BHBA	β-Hydroxybutyrate
BW	Body weight
c	*Cis*-configuration
CART	Classification and Regression Tree
CF	Crude fiber
CL	Crude fat
CLA	Conjugated linoleic acid
CoA	Coenzyme A
CP	Crude protein
CPT-1	Carnitine palmitoyl transferase 1
DHA	Docosahexaenoic acid (C22:6c4,c7,c10,c13,c16,c19)
DIM	Days in milk
DLG	Deutsche Landwirtschafts-Gesellschaft e.V.
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ECM	Energy-corrected milk yield
ENET	Elastic net
EPA	Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)
FA	Fatty acid
FPR	Milk fat-to-protein-ratio
FSH	Follicle stimulating hormone
GC	Gas chromatography
GfE	Gesellschaft für Ernährungsphysiologie
GH	Growth hormone
GLMs	Stepwise variable selection with General Linear Models
GLUT1	Glucose transporter 1
GnRH	Gonadotropin releasing hormone
HF	Holstein Frisian
IGF-1	Insulin-like growth factor 1
ISO	International Organization for Standardization
LA	Linoleic acid (C18:2c12,c15)
Lasso	Least absolute shrinkage and selection operator
LCFA	Long-chain fatty acid
LH	Luteinizing hormone

LKV BW	Landesverband Baden-Württemberg für Leistungs- und Qualitätsprüfungen in der Tierzucht e.V.
LKV NRW	Landeskontrollverband Nordrhein-Westfalen
MAX	Maximum value
MCFA	Medium-chain fatty acid
ME	metabolizable energy
MIN	Minimum value
MIR	Mid-infrared spectroscopy
ML	Maximum likelihood
MPR BW	Milchprüfring Baden-Württemberg
MUFA	Monounsaturated fatty acid
MY	Milk yield
MYs	Milk yield at the day of sampling
MYw	Mean milk yield of the week prior to sampling
n-3	*Omega-3* fatty acid
n-6	*Omega-6* fatty acid
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NEL	Net energy for lactation
NRC	National Research Council
OA	Oleic acid (C18:1c9)
OCFA	Odd-chain fatty acid
OM	Organic matter
OOB	Out-of-Bag
PUFA	Polyunsaturated fatty acid
r	Pearson correlation coefficient
R^2	Coefficient of determination
R^2_{adj}	Adjusted coefficient of determination
REML	Restricted maximum likelihood
Ridge	Ridge regression
RMSE	Root mean square error
RNB	Ruminal nitrogen balance
SCC	Somatic cell count
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SD_{rd}	Standard deviation of the raw deviations from leave-one-out cross-validation
SCFA	Short-chain fatty acid
SFA	Saturated fatty acid
t	*Trans*-configuration
TAG	Triacylglycerol
TMR	Total mixed ration
uCP	Utilizable crude protein
UFA	Unsaturated fatty acid
VLDL	Very low density lipoproteins

1 INTRODUCTION

Energy supply of high yielding dairy cows, especially in early lactation, is one of the most challenging factors in dairy cow nutrition. Negative energy balance (NEB) in dairy cows usually originates in early lactation, when energy supply via dry matter intake (DMI) increases slower than energy expenditure for milk production. This energy deficit can differ in severity and duration depending on various factors, mainly DMI and milk yield (MY) postpartum. Energetically, the use of body tissues accounts for about 30% of milk production during the first month of lactation (Bauman and Currie, 1980; Schröder and Staufenbiel, 2006). It can take up to two months until the energy uptake matches the requirements and leads to an equilibration and then to a positive energy balance (EB; Knight, 2001; Gross et al., 2011b). From this day on, cows start restoring their energy reserves they had mobilized during NEB (Knight, 2001). This is a natural mechanism which, however, has been intensified to a problematic degree by the breeding progress of the last decades. The continuously increasing milk performance of the modern dairy cow has not been accompanied by a proportional increase of feed intake capacity which led to more severe NEB. Indirectly, this breeding progress selected cows for their ability to mobilize body reserves which in turn can impair the cow's health if performed excessively (McNamara and Hillers, 1986; Roche et al., 2009). Ketosis or the fatty liver syndrome are the most abundant disorders caused by excessive body fat mobilization, accompanied by impaired fertility (Grummer, 1993; Goff and Horst, 1997). NEB can also occur in later lactation, e.g. as a consequence of insufficient energy supply due to flawed diet formulation or feed components of minor quality.

There is an increasing interest in selecting cows for breeding which are not prone to excessive NEB, and thus to consequential metabolic disorders, but little is known about the physiological mechanisms behind this phenomenon (van Knegsel et al., 2014a). While problems with the diet formulation or feedstuff quality can be identified on herd or group level, the selection of cows prone or not prone to NEB requires monitoring of their individual energy balance (EB). However, the exact, noninvasive monitoring of EB requires information about DMI which is usually not available at commercial farms in contrast to MY. Therefore, some efforts were made to identify blood or milk indicators which point out NEB or to predict EB (i.a. Bowden, 1971; Clark et al., 2005; Gross et al., 2011a; Thorup et al., 2012; Jorjong et al., 2014).

The objectives of the present thesis were 1) to give an overview about the causes and consequences of NEB, management strategies to support cows' energy metabolism in early lactation, and factors influencing the milk fatty acid (FA) profile and 2) to predict the EB of dairy cows from various animal, diet, and milk traits with special regard to the milk FA profile. In consideration of its possible future application in monthly milk recording, this prediction was supposed to work without continuous data (e.g. daily), but with single milk samples.

2 LITERATURE REVIEW

2.1 Negative Energy Balance: Causes and Consequences

2.1.1 Metabolic Changes in Transition and Early Lactation

Transition period is usually defined as the time span of about three weeks before and after parturition (Grummer, 1995). In 1995, Grummer stated in one of the first reviews about the feeding of the transition cow that literature focusing on transition period, especially on feeding, were scarce. However, during the following two decades the transition period as well as its physiological background and risks have been subject of intense research resulting in numerous publications which again served as the basis for several reviews (Bell and Bauman, 1997; Herdt, 2000; Drackley, 1999; Drackley et al., 2001; Drackley et al., 2005; Horst et al., 2005; Ingvartsen, 2006; Remppis et al., 2011; Roche et al., 2013; van Knegsel et al., 2014a; Zebeli et al., 2015). Transition is the most critical phase for high-yielding dairy cows, as their metabolism shifts from late gestation to lactation and places high demands on management and nutrition.

With the onset of lactation the energy (and nutrient) requirements of high-yielding dairy cows increase dramatically and cannot be covered by feed intake immediately, which results in NEB (Grummer et al., 2004; Block, 2001). The animals' whole metabolism and the partitioning of nutrients have to undergo severe adjustments to match these demands. This process is called homeorhesis, defined by Bauman and Currie (1980) as "the orchestrated or coordinated changes in metabolism of body tissues necessary to support a physiological state".

The mammary gland dominates the need for nutrients postpartum: compared with the gravid uterus in late pregnancy, the mammary gland of a 30 kg milk yielding Holstein cow at four days in milk (DIM) requires about 2.7, 2.0 and 4.5 times more glucose, amino acids, and FA, respectively (Bell, 1995).

2.1.1.1 Glucose, Protein, and Calcium Metabolism

The elevated need for glucose in the mammary gland, mainly as precursor for lactose synthesis, involves an increased gluconeogenesis and glycogenolysis in the liver and a decreased use of glucose as energy source by other body tissues (Bauman and Currie, 1980; Drackley et al., 2001; Weber et al., 2013). The latter is caused by reduced plasma insulin concentrations and thus a decreased responsiveness to insulin in muscle and adipose tissue (Bauman and Elliot, 1983; Bossaert et al., 2008; Hammon et al., 2009). The lack of insulin and thus glucose in peripheral tissues prevents anabolic processes and supports mobilization of labile protein in muscle tissue and lipolysis in adipose tissue (Bauman and Currie, 1980).

Furthermore, the glucose uptake of the mammary gland does not depend on insulin concentrations in contrast to other tissues (Laarveld et al., 1981; Zhao et al., 1993). These mechanisms contribute to the glucose partitioning giving priority to the mammary gland and thereby to milk production (van Knegsel et al., 2007b).

As the majority of dietary carbohydrates is fermented to short-chain fatty acids (SCFA) in the rumen and only about 5–10% of the required glucose is absorbed by the intestinal tract, the remaining demand for glucose is to be covered by gluconeogenesis (Young, 1977; Doepel et al., 2009). The liver is the most important organ for gluconeogenesis and it was shown in sheep that the liver might perform more than 80% of the required glucose synthesis (Bergman et al., 1974). For this purpose, a sufficient supply of precursors is necessary. The dominant precursor is propionate from ruminal fermentation, which accounts for up to 70% of the hepatic gluconeogenesis, followed by lactate (15–20%), amino acids (10–15%, mostly alanine), and glycerol (2–4%) from lipolysis (Danfær et al., 1995; Reynolds et al., 2003; Larsen and Kristensen, 2009). However, as the propionate supply is dependent on ruminal fermentation and hence on DMI, there is a shift to an enhanced use of lactate from the Cori Cycle and amino acids around parturition because the reduced DMI at this time leads to a lower propionate supply (Bell et al., 2000; Reynolds et al., 2003; Hammon et al., 2009). Even so, this shift is reversed with proceeding lactation.

The decline in DMI, the use of amino acids for gluconeogenesis, and the severely increased milk protein output with the onset of lactation also lead to a deficiency in amino acid supply and thus to a negative protein balance (Doepel et al., 2009). Therefore, in early lactation also labile protein sources in skeletal muscle tissues are mobilized to serve as substrate for gluconeogenesis and even more for milk protein synthesis (Bauman and Currie, 1980; Bell et al., 2000). This process was observed to start prepartum, in advance of body fat mobilization, and to last with a postpartum decline until three to five weeks of lactation (Doepel et al., 2002; Kokkonen et al., 2005; van der Drift et al., 2012). The increasing supply with amino acids due to increasing DMI seems to influence the decline in peripheral protein mobilization (van der Drift et al., 2012). In support of this, Tesseraud et al. (2007) demonstrated that increasing insulin levels inhibit the proteolysis with ongoing lactation in dairy goats.

Calcium metabolism is also affected by the onset of lactation and the cow's requirement increases by up to more than 400% from the day of parturition (Goff and Horst, 1997). The homeostasis of blood calcium is under strict hormonal regulation. The mechanisms include increased release of parathyroid hormone and renal release of active vitamin D_3 metabolites to support intestinal calcium absorption, reduced urinary calcium excretion, and calcium mobilization from bones (Bauman and Currie, 1980; Horst et al., 2005).

2.1.1.2 Lipid Metabolism

The enhanced energy requirement also causes changes in lipid metabolism. Aside from the mammary gland during lactation, the liver and the adipose tissue are the most important organs concerning lipid metabolism (Drackley et al., 2001; Vernon, 2005). Lipolysis and lipogenesis always occur simultaneously in adipocytes and the metabolic status of the animal determines which process predominates (McNamara, 1995). After parturition, lipolysis clearly dominates the adipocyte metabolism (Bauman and Currie, 1980; McNamara, 1991). Body fat reserves are mobilized from adipose tissue to meet the energy requirements. Triacylglycerols (TAG) stored in adipocytes are hydrolyzed and the resulting non-esterified fatty acids (NEFA) and glycerol are released into the blood (Drackley, 1999; Chilliard et al., 2000a).

This process is mainly subject to hormonal regulation. Catecholamines, for example, stimulate lipase activity and thus lipolysis in the short term. The responsiveness of the adipose tissue to catecholamines increases prepartum and remains elevated throughout lactation (McNamara and Hillers, 1986). Normally, insulin increases lipogenesis and the activity of lipoprotein lipase, but the low insulin plasma concentrations during transition and the reduced responsiveness of the adipose tissue to insulin cause a restraint in lipogenesis and support lipolysis (Chilliard et al., 2000a; Hayirli, 2006). In terms of homeorhesis growth hormone (GH) modulates adipose tissue metabolism by amplifying the effects of catecholamines and extenuating insulin effects (Chilliard et al., 2000a; Vernon, 2005). However, the adipose tissue also underlies self-control. Among others, it secretes peptide hormones termed adipocytokines, including leptin, resistin, tumor necrosis factor α, interleukin-6, and adiponectin, which can modulate the adipose tissue metabolism and also the metabolism of other tissues. Their secretion increases with adipose tissue mass, except for adiponectin (Vernon, 2005). Leptin, which is almost exclusively produced by adipose tissues, affects the hypothalamic metabolism causing decreased DMI (Pittas et al., 2004; Sartin et al., 2011). Tumor necrosis factor α and interleukin-6 can also have an appetite decreasing effect and additionally support insulin resistance together with resistin and leptin (Fasshauer and Paschke, 2003; Pittas et al., 2004). In contrast, adiponectin supports insulin effects and helps reducing TAG concentration in other tissues (Fasshauer and Paschke, 2003; Giesy et al., 2012).

In the liver GH causes the release of insulin-like growth factor 1 (IGF-I), which in turn has a negative feedback on GH secretion from the pituitary (Lucy et al., 2001). In transition, though, the expression of hepatic receptors for GH is reduced, which is believed to lead to the increased GH and decreased IGF-I blood levels (Lucy et al., 2001). This is associated with an intensification of the direct catabolic effects of GH on adipose tissue and its diminished anabolic effects on peripheral tissues (Lucy et al., 2001).

The increasing plasma NEFA concentrations and the elevated blood flow contribute to matching the increased energy requirements in early lactation by additional supply of NEFA to the liver and other tissues for oxidation and thus energy generation, concurrently sparing glucose for the mammary gland (Reynolds et al., 2003; van Knegsel et al., 2007b). The liver is a central transfer site in lipid metabolism (Figure 1). It either esterifies NEFA back to TAG and then charges very low density lipoproteins (VLDL) or decomposes NEFA to acetyl coenzyme A (CoA) in the course of β-oxidation which is then used for energy yield via the Krebs cycle (Zammit, 1990). NEFA are activated by esterification with coenzyme A to acyl-CoA which is then formed to acyl carnitine and transported into mitochondria for β-oxidation. If the β-oxidation is not completed, for example due to a lack of oxaloacetate, acetyl-CoA can be formed to acetoacetate and released into the cytosol where it can be dehydrogenated to β-hydroxybutyrate (BHBA; Zammit, 1990; Drackley et al., 2001). Both are then released into the blood and can be utilized by other tissues. Consequently, increased ketogenesis during transition might additionally be a strategy to compensate for the lack of precursors for gluconeogenesis. (Drackley et al., 2001)

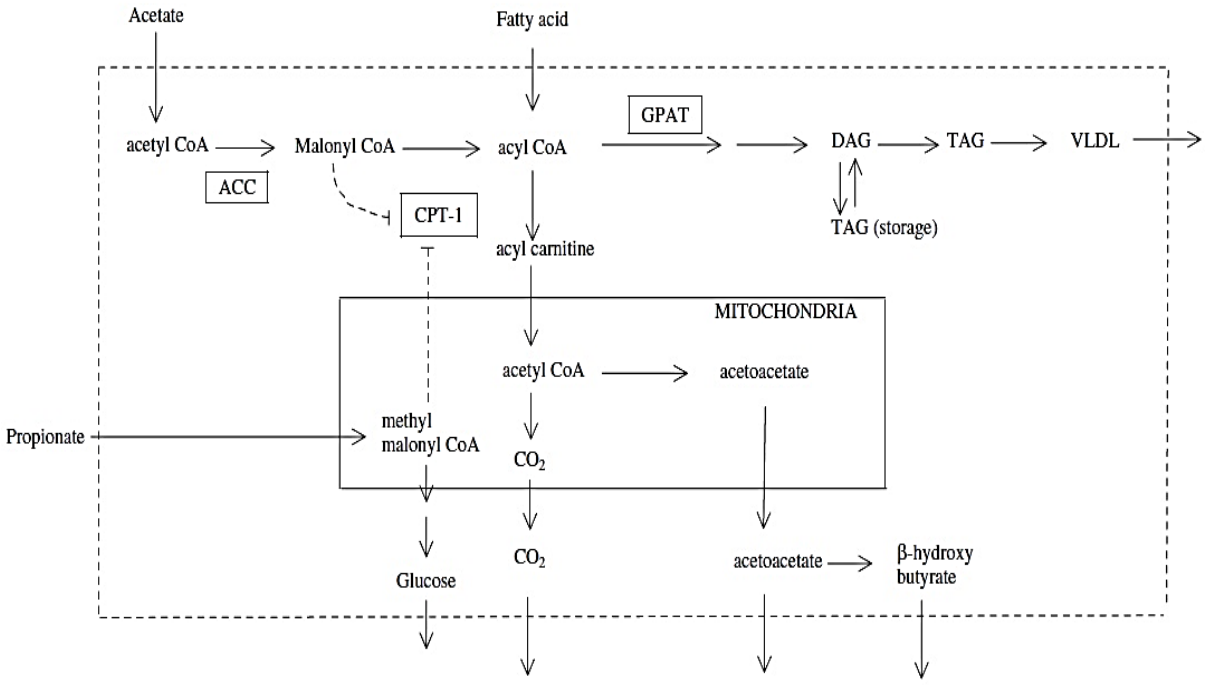

Figure 1 Hepatic lipid metabolism. ACC = acetyl-CoA carboxylase; CPT-1 = carnitine palmitoyl transferase 1; DAG = diacylglycerol; TAG = triacylglycerol; GPAT = glycerol-3 phosphate dehydrogenase; VLDL = very low density lipoproteins. Solid lines indicate metabolic pathways; dashed lines indicate allosteric inhibition. Adapted from Vernon (2005), reproduced with permission of Cambridge University Press.

During lactation, particularly in early lactation, several mechanisms stimulate the β-oxidation of FA in mitochondria. CPT-1 (carnitine palmitoyl transferase 1) catalyzes the formation of acylcarnitine. It is inhibited by malonyl-CoA which arises from the de novo synthesis of FA from acetate and methylmalonyl-CoA, an intermediate of gluconeogenesis from propionate. The low plasma insulin and high plasma NEFA levels cause a reduction of acetyl-CoA carboxylase activity, which turns acetyl-CoA to malonyl-CoA, and lead to decreasing malonyl-CoA levels in hepatocytes. Additionally, the decreased propionate supply in early lactation leads to decreasing methylmalonyl-CoA concentrations (Drackley, 1999; Dann and Drackley, 2005; Vernon, 2005). This results in an abolishment of the CPT-1 inhibition and thus enhances FA oxidation (Vernon, 2005).

Aside from the NEFA supply and CPT-1 activity, hepatic ketogenesis also depends on the activity of 3-hydroxy-3-methylglutaryl-CoA synthase which is responsible for the conversion of acetyl-CoA to acetoacetate (Hegardt, 1999). Its activity in turn is negatively correlated with propionate supply (Zammit, 1990). Ketogenesis is suspected to support hepatic gluconeogenesis, as the export of acetoacetate from mitochondria into cytosol seems to happen in exchange of pyruvate import, which can then be turned into oxaloacetate (Zammit, 1990).

The excessive hepatic NEFA uptake also causes a marked increase in esterification to TAG, which may be due to an exceedance of β-oxidation capacity (Drackley et al., 2001). Grum et al. (1996) found a 188% and a 124% increase in palmitic acid esterification in the liver on 1 and 21 DIM compared with three weeks prepartum.

Another mechanism helpful in times of extensive NEFA mobilization is peroxisomal β-oxidation, taking place in organelles named peroxisomes (Singh, 1997). In peroxisomes, FA are partially oxidized which leads to less energy gain and more heat production (Singh, 1997). Grum et al. (1996) reported that 50% of the first cycle of palmitic acid oxidation was performed by hepatic peroxisomes in early lactating cows. This pathway was shown to be induced in rodents by starvation or dietary fat, for example (Singh, 1997). It seems to serve as a kind of an “overflow” pathway in times of excessive NEFA mobilization and preferably very long chain FA (>C22) are partially oxidized which are poor substrates for mitochondrial oxidation (Adewuyi et al., 2005).

Body fat mobilization and hepatic lipid metabolism are not only a matter of insufficient energy supply but also of individuality. Kessel et al. (2008) compared metabolic parameters in transition cows (n = 54) kept under equal conditions. They retrospectively distinguished two groups: cows which showed elevated plasma BHBA (>1.0 mM) at least once during the experiment (2 weeks prepartum until 14 weeks postpartum) and cows which did not. Though

the blood metabolites showed marked differences and decreases of body weight (BW), body condition score (BCS), and backfat thickness were greater in animals with elevated plasma BHBA, EB as well as milk performance were comparable for both groups. Hammon et al. (2009), van Dorland et al. (2009), and Weber et al. (2013) investigated aspects of hepatic metabolism and their effects on the whole metabolism in transition cows. They also found individual differences in liver fat content, plasma NEFA and BHBA concentrations. This is important: it shows that excessive body fat mobilization, high liver fat content or BHBA concentrations are not inevitably consequences of mistakes in farm management (Hammon et al., 2009). Additionally, McCarthy et al. (2015) found low correlations between NEFA and BHBA concentrations in periparturient cows and caution against trying to extrapolate from one metabolite to the other.

2.1.2 Disorders Related to Negative Energy Balance

All these metabolic adaptations mentioned before are essential for a successful transition. If the cow's ability to adapt is impaired, a number of typical periparturient health problems like fatty liver syndrome, ketosis, hypocalcaemia, inflammations or limited fertility can occur. These disorders are directly or indirectly associated to NEB. But it is often not clear whether NEB is cause or consequence of the respective disorder or both (Esposito et al., 2014; van Knegsel et al., 2014a). Each disorder has an impact on DMI and MY, which in turn affect EB. Bareille et al. (2003) calculated DMI decreases for various disorders and concluded that per kg DMI decrease a mean loss of MY of 1.9 kg followed.

However, cows can tolerate a certain state of undernutrition in early lactation (Butler and Smith, 1989). The crucial factors for establishing NEB related disorders seem to be the magnitude of NEB and its duration (Collard et al., 2000; Butler, 2003).

2.1.2.1 Hypocalcaemia

As mentioned before, the calcium requirements increase enormously with the onset of lactation. If the mechanisms for homeostasis of blood calcium concentrations fail to provide the required amounts of calcium from intestinal absorption, renal reabsorption, and mobilization from the bones, hypocalcaemia might occur (Goff, 2006). This usually happens within 24 h after parturition, directly after milk release (Roche et al., 2013). As a result, muscular and nervous function is impaired, which can lead to paresis if clinical hypocalcaemia, also known as milk fever, occurs (Goff and Horst, 1997). Reinhardt et al. (2011) reported more than 40% of fresh cows in second lactation suffer from subclinical hypocalcaemia with increasing incidence up to the fifth lactation.

Clinical and subclinical hypocalcaemia are associated with decreased milk production caused by secondary diseases, like impaired function of smooth muscle tissues (Curtis et al., 1985; Goff and Horst, 1997; Goff, 2008). This causes lower motility of the gastro-intestinal tract, which can be followed by left displaced abomasum or lead to decreased DMI and then ketosis (Curtis et al., 1985; Goff, 2008). Mean total DMI decreases until recovery are estimated to 38 kg, which results in a loss in MY of about 45 kg (Bareille et al., 2003). A poor teat sphincter closure caused by hypocalcaemia predisposes for mastitis which can also contribute to decreased milk production (Curtis et al., 1985). Low uterine motility, possibly combined with cortisol secretion, can cause dystocia, retained membranes and delayed uterine involution, which may result in metritis and impair fertility (Curtis et al., 1985; Drackley et al., 2005; Bicalho et al., 2014).

Hypocalcaemia is no direct consequence of NEB but it can contribute indirectly to severity and duration of NEB.

2.1.2.2 Ketosis

In times of massive body fat mobilization, an insufficient supply of carbohydrates to metabolize acetyl-CoA leads to enhanced hepatic ketogenesis (Drackley et al., 2001; Roche et al., 2013). According to the physiological mechanisms in lipid metabolism described above, NEFA and BHBA concentrations in blood are often used as indicators for body fat mobilization (and thus energy deficit) and ketogenesis (Bowden, 1971; Adewuyi et al., 2005). Recently, Zhang et al. (2016) found alterations in carbohydrate and lipid metabolism and an activated innate immunity weeks prior to onset of ketosis in transition dairy cows. They suggested serum Interleukin-6 as a potential new biomarker for ketosis disposition of dry cows, which has yet to be further investigated.

If ketogenesis exceeds the capacity of other tissues to utilize ketone bodies for energy supply, ketones (BHBA, acetoacetate, and acetone) accumulate in the blood. This state is named ketosis. It can occur clinically or, more frequently, subclinically. In a study of Duffield et al. (1998) the incidence of subclinical ketosis (here defined by blood BHBA >1.2 mM) amounted to about 30% within the first two weeks of lactation in control cows, McArt et al. (2012a) even reported 44%. Ketosis is associated with the susceptibility for other disorders like displaced abomasum, metritis, reduced fertility, and also reduced MY (Duffield et al., 1999, Ospina et al., 2010a, 2010b; McArt et al., 2012b). On the other hand, it may also be caused by a sudden reduction of DMI due to preceding diseases (e.g. hypocalcaemia, displaced abomasum, locomotive disorders), sudden feed restriction, or from inadequate feed quality (Ingvartsen, 2006; Roche et al., 2013).

Ketosis often follows on the fatty liver syndrome, which might be caused by reduced gluconeogenesis and thus decreasing blood glucose levels. This leads to amplified body fat mobilization and consequently further increases ketogenesis (Drackley et al., 2005). Poor quality silage with high amounts of butyrate or feedstuffs like sugar beets, which cause high ruminal butyrate production (= ketogenic feedstuffs), may also contribute to ketogenesis, as they increase ruminal butyrate concentration (Ingvartsen, 2006; Roche et al., 2013). Butyrate serves as a precursor for ketone bodies – at least 50% of ruminal butyrate is converted to BHBA within the rumen epithelium, while the remains are either oxidized in the liver or used for hepatic ketogenesis (Ingvartsen, 2006).

The incidence of ketosis is not only a matter of NEB but also a matter of individual variation in metabolic adaptation to lactation (Kessel et al., 2008). Cows with high body condition (see section 2.1.2.7) carry a much higher risk to suffer from ketosis than lean animals (Grummer, 1993, 2008; Drackley et al., 2005; Ingvartsen, 2006). The risk also increases with parity and when ketosis already occurred in the past (Ingvartsen, 2006).

2.1.2.3 Fatty Liver

The hepatic release of TAG in the form of VLDL is very low in ruminants compared to other species. This leads to an accumulation of TAG in the liver of transition cows if NEFA esterification to TAG exceeds TAG hydrolysis and VLDL export capacity (Drackley et al., 2001; Vernon, 2005). It was even shown in vitro that high NEFA concentrations inhibit the production of VLDL in bovine hepatocytes and hence directly contribute to TAG accumulation (Liu et al., 2014). If this TAG accumulation occurs excessively due to extreme plasma NEFA concentrations and thus hepatic uptake in times of NEB, it can result in the fatty liver syndrome (Grummer, 1993; Drackley et al., 2001; Vernon, 2005). According to Bobe et al. (2004), a normal liver fat content is lower than 1% of wet weight, while moderate and severe fatty livers contain up to 10% and more than 10% TAG, respectively. In early lactation, a mild to moderate fatty liver is considered as physiological due to the inevitable body fat mobilization and often occurs subclinically (Ingvartsen, 2006). Various studies showed that more than 50% of dairy cows suffer from fatty liver after calving: Jorritsma et al. (2001) discovered that 54% of animals at nine commercial Dutch dairy farms had liver fat contents of >5% postpartum, while Grummer (1993) found that 50% of the animals in a meta-analysis of control groups from three different feeding trials (standard dry cow diets prepartum, normal body condition) even developed liver fat contents of >15%. Furthermore, it was reported that cows which developed a fatty liver often already showed enhanced liver fat contents before calving (Bertics et al., 1992; Grummer, 1993).

There is a positive relationship between increasing liver fat content and urinary ketone concentration, a negative one to health status and fertility, but milk production and DMI seem not to be impaired until a severe fatty liver occurs (Jorritsma et al., 2000; Jorritsma et al., 2001; Bobe et al., 2004). The disease pattern is rather diffuse: usually, sick cows show non-specific symptoms like reduced DMI, BW loss and apathetic behavior (Ingvartsen, 2006).

The occurrence of fatty liver also predisposes for secondary diseases and disorders. It often precedes ketosis, is associated with impaired liver function (and thus reduced gluconeogenesis), displaced abomasum, and reduced reproductive performance (Drackley, 1999; Drackley et al., 2001; Grummer, 1993; Jorritsma et al., 2000; van Winden et al., 2003; Ohgi et al., 2005). A fatty liver itself is mostly a reversible state and does not by all means cause permanent damage (Ingvartsen, 2006). The development of fatty livers is more likely in over-conditioned cows (Ohgi et al., 2005; Hammon et al., 2009; Roche et al., 2013), but aside from this it seems also to be a very individual occurrence. Hammon et al. (2009) found great differences in fat mobilization and liver fat content in high yielding transition dairy cows which were kept under the same conditions and offered the same diets ad libitum. In this study MY was not affected by liver fat content – which leads to the conclusion that animals use different strategies concerning nutrient partitioning. These findings support the findings of Kessel et al. (2008) described above.

Fatty liver as well as ketosis are consequences of NEB, but they also cause further decrease and/or prolonged duration of NEB by either inducing reduced DMI or secondary disorders which do so.

2.1.2.4 Immune Dysfunction

Transition is also accompanied by immune depression and increased incidence of inflammations, which contribute to an impaired disease resistance (Goff, 2006; Sordillo and Aitken, 2009; Roche et al., 2013). The immunosuppression is characterized by low immune-cell concentrations in blood and decreased immune-cell responses (Mallard et al., 1998; van Knegsel et al., 2007a; Esposito et al., 2014). The decrease in immune function was found to already start about three weeks prepartum, before the EB becomes negative (van Knegsel et al., 2014a). However, metabolic stress (here NEB) and metabolic disorders contribute to and also prolong immune dysfunction, as high plasma NEFA and BHBA as well as low plasma glucose concentrations impair immune cell activity (Lacetera et al., 2004; Sordillo et al., 2009; Roche et al., 2013). Then again, infections which manifest due to the reduced immune function and following inflammatory responses can further weaken the animal, enhance energy requirements for immune response, and thus may intensify NEB (van Knegsel et al., 2014a).

2.1.2.5 Oxidative Stress

Another factor compromising health during transition which has not been subject of extensive research yet is oxidative stress (Jóźwik et al., 2012; van Knegsel et al., 2014a). It is a result of a deficiency of antioxidants compared to the production of reactive oxygen species (Sordillo and Aitken, 2009). Susceptibility to oxidative stress is linked to NEB (Bernabucci et al., 2005; Pedernera et al., 2010) and oxidative stress may also impair liver function (van Knegsel et al., 2014a). Its impact on liver fat metabolism seems to be a reduction of apolipoprotein B expression, which binds lipids to form lipoproteins (van Knegsel et al., 2014a). This leads to decreased secretion of VLDL and thus increased hepatic TAG accumulation (van Knegsel et al., 2014a). Oxidative stress may also promote other metabolic disorders and impair immune function (Bernabucci et al., 2005; Sordillo and Aitken, 2009). But these mechanisms still have to be researched (van Knegsel et al., 2014a).

2.1.2.6 Fertility

Fertility has considerably declined simultaneously to the intense genetic selection for milk performance during the last decades (Kanitz et al., 2003; Walsh et al., 2011). But the common hypothesis that there is a clear genetic antagonism between reproductive and milk performance has recently been put into question (LeBlanc, 2010; Bello et al., 2012). Management issues or metabolic disorders may be the greater challenge for fertility (Drackley and Cardoso, 2014). NEB is associated with reduced reproductive performance, mainly delayed or irregular cycles, which contribute to reduced conception rates and an increased calving to conception interval (Butler and Smith, 1989; Patton et al., 2007; Wathes et al., 2007; Walsh et al., 2011). The nadir of EB seems to be the most important factor concerning the delayed start of luteal activity (Beam and Butler, 1999; de Vries and Veerkamp, 2000). The relation of lipid metabolism to fertility in the dairy cow has been thoroughly summarized by Wathes et al. (2012).

Usually, cows start ovulating again between 20 and 30 days postpartum and are inseminated after one or two regular cycles (Wathes et al., 2007). In phases of severe NEB, there is no spare energy for the establishment of pregnancy, thus ovarian functions are impaired by several metabolic factors (Knight, 2001). The low plasma insulin, glucose, and leptin concentrations report energy deficiency to the central nervous system which reacts by decreasing the release of gonadotropin releasing hormone (GnRH), which in turn leads to decreased secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH; Kanitz et al., 2003). FSH stimulates follicular growth, while LH induces ovulation. Additionally, the low Insulin and IGF-I levels do not promote follicular proliferation sufficiently (Kanitz et al., 2003; Wathes et al., 2007). High milk performance due to elevated prolactin and GH concentrations

also results in lower GnRH and thus lower FSH and LH secretion (Kanitz et al., 2003). High NEFA concentrations are also associated with a decreased oocyte quality (Leroy et al., 2005).

Uterine health problems like retained placenta, metritis, and endometritis are all known to impair fertility (Roche, 2006; Fourichon et al., 2000; Wathes et al., 2007). Immune functions in the uterus are suppressed during pregnancy (amongst others by progesterone) in order to protect the conceptus from the maternal immune system (Esposito et al., 2014). After calving, the uterus is particularly susceptible to infections, which seems inevitable to a certain extent (Walsh et al., 2011; Esposito et al., 2014). More than 80% of the animals examined show uterine bacterial contamination during the first two weeks postpartum (Sheldon et al., 2006). NEB can cause or worsen these problems and lead to a prolonged healing process due to its adverse effects on immune function (Wathes et al., 2007). Metabolic disorders associated with elevated blood NEFA and BHBA concentrations like fatty liver or ketosis may also contribute to a poor reproductive performance (Jorritsma et al., 2000; Ospina et al., 2010a; Drackley and Cardoso, 2014).

2.1.2.7 Risk Factor Body Condition

The cow's body condition is a very important factor concerning her susceptibility to metabolic disorders. There is plenty of evidence that especially over-conditioned cows rather suffer from metabolic disorders and poor fertility than animals which are in optimal body condition (Treacher et al., 1986; Rukkwamsuk et al., 1998; Ingvartsen, 2006; Roche et al., 2009; Remppis et al., 2011). Additionally, the extent of body condition loss postpartum is known to affect health and fertility (Kim and Suh, 2003). The estimation of body reserves in the form of adipose and muscle tissue is usually performed using BCS according to Edmonson et al. (1989). The animal's body reserves are determined by visual evaluation and/or palpation of certain body parts and classified by means of a five-point-scale, where 1 means extreme malnutrition and 5 severe over-conditioning (Edmonson et al., 1989). This procedure is easy and practical but also very subjective. Therefore, it is recommended to be performed by the same qualified person if possible (Roche et al., 2009; Aktas et al., 2011). The changes in BCS over time can give information about whether the cow is mobilizing body reserves due to NEB or replenishing her reserves as a result of excess energy supply. Body weight or its changes are inappropriate indicators for animals' body condition as both also depend on frame size and permanently changing gut fill (Wildman et al., 1982). Another method for determining the body condition is the ultrasonographic measurement of backfat thickness at a defined spot on an imaginary line between the tuber coxae and tuber ischia (Schröder and Staufenbiel, 2006). One millimeter of backfat corresponds to approximately 5 kg of body fat (Klawuhn and Staufenbiel, 1997). This method is less subjective than BCS but it is mainly used for scientific purposes as a portable ultrasound unit is required.

A crucial factor for a cow's successful transition seems to be the body condition at calving (Roche et al., 2009). In general, it is recommended that cows should enter the dry period when they already achieved the target BCS for calving (Grum et al., 1996). Depending on the literature source, values of 3.0 to 3.75 are considered as an optimal BCS for Holstein Frisian (HF) cows at parturition (Grum et al., 1996; Roche et al., 2009; Drackley and Cardoso, 2014). The recommendations seem to decrease over time: Wildman et al. (1982) recommended an optimal calving BCS of 3.5 to 3.75, while recently Roche et al. (2009) and Drackley and Cardoso (2014) advised 3.0 to 3.25. This may be a consequence of the intense genetic selection for MY which led to leaner animals (Drackley and Cardoso, 2014). On this account, there are also different recommendations for breed, with higher values for dual purpose than for pure milk breeds.

As mentioned before, over-conditioned cows are far more likely to develop ketosis and fatty liver. Thus the clinical appearance of fatty liver is also known as the "fat cow syndrome" (Butler and Smith, 1989). Gillund et al. (2001) observed that cows with a calving BCS of 3.25 bear half the risk of suffering from ketosis than cows having a calving BCS of >3.5. This is due to the fact that over-conditioned cows have lower DMI, which is likely to be induced mainly by leptin, the concentration of which increases with body fatness (Kokkonen et al., 2005; Chilliard et al., 2001a). Consequently, more lipid reserves are mobilized with the onset of lactation in order to support milk production, which then leads to an amplified accumulation of TAG in the liver and ketones in blood (Grummer, 1993; Broster and Broster, 1998; Weber et al., 2013). Additionally, there is a negative relationship between the level of over-conditioning prepartum and the insulin response of the glucose metabolism (de Koster et al., 2015). The low DMI and enhanced tissue mobilization also lead to a prolonged and more intense loss of BCS in early lactation (Garnsworthy and Topps, 1982; Broster and Broster, 1998; Holtenius et al., 2003). On the other hand, there was no difference in MY in over-conditioned cows, as they can compensate their reduced energy intake mainly by the increased mobilization of energy reserves (Agenäs et al., 2003). Garnsworthy and Topps (1982) reported from the second of two identical trials, during which three groups of cows were fed prepartum to low (1.5–2.0), medium (2.5–3.0) and high (3.5–4.0) BCS at calving, that the low BCS group produced more milk than the others. However, there was no difference in MY in the first trial. They even concluded that there seemed to be no advantage in feeding cows to achieve a BCS greater than 2.0 at calving as thinner cows can produce more milk from feed due to their higher DMI. Treacher et al. (1986) also found no difference in total MY in cows with a calving BCS of either 2.8 or 3.9, but they reported significantly higher MY in lower conditioned cows during the first six weeks of lactation. It has to be taken into account that, more than 30 years ago, these animals had a peak MY of about 26 to 30 kg (Garnsworthy and Topps, 1982) and 29 to 34 kg (Treacher et al., 1986). Consequently, it might have been easier for those "ancient" cows to

compensate the energy deficiency and even improve MY by increasing DMI than for today's high yielding cows giving up to 50 kg milk per day in early lactation.

Such a low calving BCS as recommended by Garnsworthy and Topps (1982) can have detrimental effects on fertility, though. Their trial showed better results (numerically for trial 1, significantly for trial 2) for moderate BCS concerning the days to first observed estrus, days to conception, and number of inseminations compared with low BCS. In general, it seems that thin cows have a higher first service conception rate but a longer anestrus interval after calving (Remppis et al., 2011). Nonetheless, also in terms of fertility over-conditioning and especially excessive loss of condition postpartum seem to cause the greater complications. Many studies proved high body condition and excessive condition loss to negatively influence reproductive parameters like days to first estrus, days to first breeding, conception at first insemination, days open and number of inseminations (Garnsworthy and Topps, 1982; Wildman et al., 1982; Gillund et al., 2001; Pryce et al., 2001; Kim and Suh, 2003; Roche, 2006; Roche et al., 2009).

According to Roche and Berry (2006), who investigated influential factors for milk fever in grazing dairy cows, the risk for milk fever is also enhanced in cows with a BCS <2.50 and >3.50. A possible explanation for over-conditioned cows might be an insufficient level of DMI and thus calcium intake directly postpartum. The finding that fat cows rather suffer from clinical milk fever is supported by Heuer et al. (1999) and Stockdale (2007), while Stockdale additionally found a higher incidence of subclinical milk fever in thin cows than in fat ones. Additionally, Bernabucci et al. (2005) reported that cows with a BCS >3.0 and greater BCS losses postpartum are more likely to experience oxidative stress than others.

2.1.3 Dry Matter Intake

DMI is considered the most limiting long term factor in milk production (Allen, 2000). While milk performance is mainly a matter of genetic determination, DMI is also the most susceptible factor contributing to EB (Allen, 2000).

The regulation of DMI is very complex and can be affected by factors at the cellular level up to environmental conditions (Allen, 2000). The mechanisms of hunger and satiety and their influence during the transition period were intensely investigated and reviewed during the last decades but are still not fully understood (Allen, 2000; Ingvartsen and Andersen, 2000; Ingvartsen and Boisclair, 2001; Grummer et al., 2004; Hayirli and Grummer, 2004; Roche et al., 2008; Sartin et al., 2011; Kuhla et al., 2016). The center of feed intake regulation and energy expenditure is the central nervous system, to be more exact, the hypothalamus (Ingvartsen and Boisclair, 2001). Information about the metabolic state is provided to the hypothalamus by stimulation of the vagus nerve, metabolites (NEFA, BHBA), or multiple

messenger substances secreted mainly by the adipose tissue (leptin), the gastro-intestinal tract (cholecystokinin, ghrelin), and the pancreas (insulin, glucagon; Roche et al., 2008; Sartin et al., 2011). These signals can have orexigenic or anorexigenic effects, and operate either according to the acute energy status or for chronic control (Roche et al., 2008).

The energy metabolism of adult animals in general seems eager to maintain a certain amount of body reserves, a certain body condition, which is named set point (Harris, 1990). In case the body condition exceeds or falls short of this set point, metabolic mechanisms are activated which are supposed to adjust to the desired status (Harris, 1990). These mechanisms are responsible for lower DMI in over-conditioned cows and higher DMI in thin cows until this set point is achieved. For example, leptin concentrations in blood increase with increasing adipose tissue mass and are thus jointly responsible for low DMI in fat animals (Chilliard et al., 2001a).

Thus, feed intake usually is adjusted to the energy requirements and the available energy reserves. In contrast to this principle, the DMI of dairy cows declines by approximately 20 to 40% during the last two weeks of gestation, although energy requirements increase (Bertics et al., 1992; Bell, 1995; Roche et al., 2008). Additionally, the postpartal DMI increases delayed to the increasing energy expenditure caused by milk production (Bell, 1995). It was assumed that the prepartal decline in DMI is induced by limited abdominal capacity due to the growing fetus, but metabolic changes also seem causative (Ingvartsen and Boisclair, 2001). Ingvartsen and Andersen (2000) suspected that elevated plasma estrogen concentrations and the onset of body fat mobilization, which lead to elevated NEFA and BHBA concentrations, contribute to the decreased DMI. Leptin, which is secreted by the adipose tissue and has an intake limiting effect, may contribute to limited DMI during late gestation (Ingvartsen and Boisclair, 2001; Pittas et al., 2004). However, leptin is unlikely to play a role directly prepartum, as its concentrations in serum decrease around parturition, partly affected by the low insulin levels (Ingvartsen and Boisclair, 2001; Leury et al., 2003; Reist et al., 2003). This drop in plasma leptin concentrations during transition is believed to be caused by NEB (Block, 2001). Block (2001) showed that cows which were not milked after parturition had double leptin concentrations than cows which were milked and therefore were in NEB. Leptin remains low in early lactation (at 50% of former prepartal concentrations) which might help increasing DMI aside from various other endocrine factors until it matches energy requirements (Ingvartsen and Andersen, 2000; Block, 2001).

One primary goal of transition cow management is keeping the prepartal decline in DMI as low as possible while promoting the increase of DMI, and thus energy intake, postpartum as far as possible to prevent a long and severe NEB and body fat mobilization.

2.1.4 Transition Feeding

As shown above, NEB can be cause as well as consequence of several disorders which, in turn, can trigger and aggravate each other and NEB, respectively. Therefore, the most effective way to facilitate successful transition seems to be improving the energy supply and facilitating the metabolic changes, which means preventing excessive fat mobilization, minimizing NEB-nadir, and shorten NEB duration. The main instrument to achieve this goal is the nutritional management, both, pre- and postpartum, which has been reviewed comprehensively by several authors (Grummer, 1995; Stockdale and Roche, 2002; Friggens et al., 2004; Overton and Waldron, 2004; Beever, 2006; Remppis et al., 2011; Roche et al., 2013; Drackley and Cardoso, 2014; Zebeli et al., 2015). In the following, some examples of dietary management strategies during transition are presented, for detailed information the reader is referred to the aforementioned review articles.

2.1.4.1 Diet Formulation

Prepartal Plane of Nutrition

The first important step for successful transition is the cow entering the dry period in optimal body condition, as discussed above. In early dry period, energy supply is recommended to be restricted, aiming to minimize the gain of body condition (NRC, 2001). Daily energy requirements for a cow having a BW of 650 kg are approximately 51 MJ NEL in far-off and 56 MJ NEL in close-up dry period (GfE, 2001). In general, over-consumption of energy during the whole dry period, compared with slightly restricted or need-based energy supply, seems to have detrimental effects on postpartal metabolism, mostly due to lower DMI and more an intense body fat mobilization (Holtenius et al., 2003; Drackley and Cardoso, 2014). Several studies found decreased DMI, thus lower EB, increased BHBA and NEFA in blood and/or a higher TAG content in overfed dry cows, even if the cows were not over-conditioned (Grummer et al., 1995; Douglas et al., 2006; Dann et al., 2006; Janovick and Drackley, 2010; Janovick et al., 2011; Mann et al., 2015; Urdl et al., 2015; Mann et al., 2016). Cows fed an energy restricted diet show higher plasma NEFA concentrations prepartum (Dann et al., 2006; Douglas et al., 2006; Janovick et al., 2011), which might help preparing the liver for the further rising NEFA flow in early lactation (Friggens et al., 2004). Additionally, the increased postpartal DMI might prevent excessive body fat mobilization. Beever (2006) concluded that the control of energy intake is necessary throughout the whole dry period and that the avoidance of luxury intakes could best be realized by feeding a high-bulk, low energy diet ad libitum (e.g. 50% chopped straw, 50% lactation ration). On the other hand, the National Research Council (NRC, 2001) recommends an energy concentration of approximately 5.2 MJ NEL/kg dry matter (DM) for dry cows until the start of the transition period, followed by 6.4 to 6.8 MJ NEL/kg DM until parturition

in order to prime the cow and her rumen for the following high performance diets. This effect can also be achieved if the cows are fed a limited, and not ad libitum, higher energy diet to avoid luxury intakes (Drackley and Cardoso, 2014). For practical purposes, however, feed restriction is hard to implement. In an experiment of Dann et al. (2006), dry cows (n = 74) were fed either to meet NRC recommendations for energy supply by 100, 150, or 80% from dry-off until 25 days prior to the expected parturition. After that, the cows received a diet either ad libitum to meet or exceed NRC recommendations or they were restricted to 80% of NRC recommendations until parturition. Their results showed that the far-off dry cow feeding management has a greater impact on performance and metabolic traits postpartum than the close-up feeding. Additionally, effects were greater for overfeeding than for feed restriction. Cows overfed in the early dry period had the lowest DMI and EB combined with the highest plasma concentrations of NEFA and BHBA during the first 10 DIM. They also had the highest incidence of health disorders (Dann et al., 2006). Winkelman et al. (2008) found no postpartal differences in ad libitum and restricted fed dry cows, except for a higher EB for the restricted group during the first week of lactation. The beneficial effects of restricted energy intake in both studies were limited to the very beginning of lactation, but this particular time span might be decisive as McArt et al. (2012b) reported that at five DIM the peak prevalence and incidence of subclinical ketosis occurs.

Fat Supplementation

Another attempt to improve cows' metabolism is feeding diets containing additional fat during transition and early lactation, aiming to provide additional energy and consequently decrease body fat mobilization and thus plasma NEFA concentrations (Kronfeld, 1982). Grum et al. (1996) compared three diets, one control containing low energy and two high-energy diets containing either high amounts of grain or fat, which were fed during the whole dry period until the 7th day prior to the expected calving. Cows fed the high-fat diet had far lower liver TAG content postpartum than cows fed other diets. But these animals also had a lower DMI and higher NEFA concentrations during the dry period (Grum et al., 1996). It was not clear whether the fat supplementation or the decreased DMI and thus a low energy intake was causative for the low liver TAG content and the elevated peroxisomal β-oxidation. Subsequent studies concluded that the decreased DMI must have been the reason for the effects on liver metabolism (Douglas et al., 2004; Douglas et al., 2006). Both studies could not show significant benefits from feeding high-fat diets to dry cows (Douglas et al., 2004; Douglas et al., 2006). Andersen et al. (2008), however, reported that a high-fat diet (8.1% CL in DM) containing supplemental saturated fat could prime the dry cow for body fat mobilization in early lactation when fed restricted according to the cow's energy requirements. Cows which were fed this diet had higher plasma NEFA concentrations prepartum but lower plasma NEFA and hepatic TAG

concentrations during the first two weeks of lactation compared with the control group to which an isoenergetic diet without additional fat was fed.

Adverse effects, which can be caused by dietary fat supplementation, are reduced DMI and impaired fiber digestion which leads to lower milk fat production, known as milk fat depression (Palmquist and Jenkins, 1980; Jenkins, 1993). The probability that ruminal fermentation might be affected increases with the degree of unsaturation and the applied amount of the dietary fat and its availability for rumen microorganisms (free or protected fat; Jenkins, 1993). Other studies confirmed the beneficial effects of saturated compared to unsaturated fat sources fed to dry cows (Moallem et al., 2007; Andersen et al., 2008). Rumen-protected fats, for example in the form of calcium salts of FA, are supposed to not impair ruminal fermentation regardless of the FAs' degree of unsaturation (Grummer and Carroll, 1991). Duske et al. (2009), however, still reported negative effects of a high-fat dry cow diet containing calcium salts of mainly palmitic acid on DMI, EB, MY, and plasma NEFA during the first four weeks of lactation and on milk fat concentration from week 5 to 14. Moallem et al. (2007) achieved similar results comparing two protected fat sources: prilled fat containing mainly saturated fatty acids (SFA) and calcium salts containing high proportions of unsaturated fatty acids (UFA).

Apparent detrimental effects can also be considered as beneficial: some researchers tried to decrease energy output by deliberately inducing milk fat depression by feeding conjugated linoleic acids (CLA) and thus improving EB of cows in early lactation and favor their metabolism: studies indeed reported lower milk fat concentrations and milk fat yields, but also increased MY which compensated the spare energy (Bernal-Santos et al., 2003; Galamb et al., 2016; Rezaei Roodbari et al., 2016) or showed no effect on MY and EB (Moore et al., 2004; Castañeda-Gutiérrez et al., 2005; Hötger et al., 2013). However, Moore et al. (2004) observed less days to EB-nadir with CLA supplementation. Galamb et al. (2016), feeding supplemental CLA either from 3 weeks prepartum (Group 1) or from calving (Group 2) until approximately 11 to 13 weeks postpartum, found less prevalence for subclinical ketosis for Group 1 compared with Group 2 and control.

Altogether, the results of feeding supplemental fat to peripartum cows are inconsistent and tend to show small or no beneficial effects at all (Grummer, 1995; Overton and Waldron, 2004).

Source of Dietary Carbohydrates

The dietary carbohydrate source may also be capable of manipulating the cows' metabolism. Glucose is generated either from glucogenic nutrients (compounds providing 3 carbon atoms) such as ruminal produced propionate and lactate, and glucogenic amino acids, or arises from starch reaching the intestine (van Knegsel et al., 2005). Lipogenic nutrients (providing 2 carbon atoms) mainly arise from dietary fiber (acetate and butyrate production in the rumen), dietary

fat, and body fat (van Knegsel et al., 2005). Lipogenic nutrients are supposed to increase the energy partitioning in favor of milk production (particularly milk fat production), while glucogenic nutrients mostly favor energy partitioning into body tissue (van Knegsel et al., 2007b). A study of van Knegsel et al. (2007b; 2007c) investigated the hypothesis that increasing the supply of glucogenic and decreasing the supply of lipogenic nutrients during transition improves EB in early lactation by antagonizing body fat mobilization and milk fat production to some extent. They compared two isoenergetic diets (approximately 6.6 MJ NEL/kg DM), containing either glucogenic or lipogenic concentrates. The diets differed mainly in their dietary carbohydrate source: the glucogenic diet contained 3.4% crude fat (CL), 53.8% neutral and acid detergent fiber, and 26.7% starch, while the concentrations for the lipogenic diet were 5.4, 65.6, and 9.5%, respectively. The results showed that cows fed the glucogenic diet indeed partitioned less energy into milk and had lower milk fat yields than cows fed the lipogenic diet (van Knegsel et al., 2007b). A tendency for higher body fat mobilization existed for lipogenic fed cows, the animals had numerically higher NEFA and lower insulin concentrations but no significant results could be obtained (van Knegsel et al., 2007c). These results were largely confirmed by comparable subsequent studies which obtained similar tendencies for a better metabolic status in animals which were fed a glucogenic diet (Piccioli-Cappelli et al., 2014; Chen et al., 2015). In accordance to this, high starch feeding in the dry period or during transition was also a strategy discussed earlier by some authors (Grum et al., 1996; Dann et al., 1999; Doepel et al., 2002). The hypothesis implied that higher starch contents would lead to production of more propionate supporting hepatic gluconeogenesis, increasing insulin secretion, and thus limiting body fat mobilization (Friggens et al., 2004). But postpartal effects were inconsistent (Friggens et al., 2004), which might have been due to insulin resistance during transition impairing the desired effect of insulin to adipose tissue (Chilliard et al., 2000a).

2.1.4.2 Supplements

Glycerol

Another possibility to improve the energy metabolism of transition cows is the direct support of gluconeogenesis via supplementation of glucogenic precursors. By way of example, glycerol is available as a byproduct of biodiesel production. On the one hand it can be converted into volatile FA, predominantly propionate, on the other hand, it can be oxidized in the liver (Paiva et al., 2016). Nonetheless, results in feeding glycerol in early lactation are heterogeneous: DeFrain et al. (2004) observed decreased DMI and blood glucose, and increased blood BHBA concentrations when supplementing glycerol by either 430 or 860 g/d. A daily supplement of 250 g in a study of Chung et al. (2007) induced no significant effects on production or metabolic traits, which could be due to the relatively low amounts of supplemented glycerol compared with DeFrain et al. (2004). However, even the replacement of corn with glycerol (approx. 11%

of dietary DM) fed to transition cows showed no significant effects postpartum (Carvalho et al., 2011). Testing different amounts of glycerol (100, 200, and 300 g/d), Wang et al. (2009) reported no effects on MY and DMI, but increased plasma glucose and decreased BHBA, which intensified with increasing glycerol level. Other studies observed increased MY (Lomander et al., 2012; Omazic et al., 2013).

Propylene Glycol

Propylene glycol is another glucogenic precursor which can be administered to dairy cows. It can contribute to gluconeogenesis in three different ways: propylene glycol is predominantly metabolized to propionate in the rumen, but it also can be absorbed and converted to lactate, or be directly transferred into the Krebs cycle via carboxylation of pyruvate into oxaloacetate (Kristensen et al., 2002; Nielsen and Ingvartsen, 2004). Nielsen and Ingvartsen (2004) published a comprehensive review dealing with propylene glycol for dairy cows and concluded that administration of propylene glycol can positively affect the carbohydrate and fat metabolism and thus decrease the risk of ketosis or fatty liver (Nielsen and Ingvartsen, 2004). Furthermore, propylene glycol generally has no effect on energy-corrected milk yield (ECM) or DMI (Nielsen and Ingvartsen, 2004). However, due to its low palatability, Nielsen and Ingvartsen (2004) recommended to mix it thoroughly with other feedstuffs or drench it, otherwise propylene glycol may negatively affect DMI, nevertheless. More recent studies focusing on propylene glycol supplementation for periparturient or early lactating cows also achieved predominantly positive results. While Moallem et al. (2007) observed no effects during transition supplementing 909 g/d of an dry additive containing 55% propylene glycol (corresponds to 500 g/d pure propylene glycol), Lomander et al. (2012), feeding 300 g/d of pure propylene glycol, observed increased MY without effects on metabolic status. Liu et al. (2009) compared the supplementation of 0, 150, 300, and 450 ml of propylene glycol per day from calving to 63 DIM. They reported no effects on DMI and MY but linearly increasing EB, blood glucose, and linearly decreasing plasma NEFA and BHBA with increasing propylene glycol administration. Drenching cows with 400 ml propylene glycol from one week prepartum until one week postpartum, Rukkwamsuk and Panneum (2010) reported lower plasma NEFA and liver TAG concentrations at two weeks postpartum compared with the untreated control group.

Monensin

Monensin is a carboxylic polyether ionophore, which is produced by *Streptomyces cinnamonensis* (Duffield et al., 2008a). It is an antibiotic which alters the ruminal microbiota by inhibiting mainly gram positive microbes (Ipharraguerre and Clark, 2003). This leads to enhanced propionate production, when gram negative microbes predominate in the rumen (Ipharraguerre and Clark, 2003). As a consequence, monensin is capable of supporting the

metabolism of the cow by helping to provide more propionate as glucogenic precursor (Ipharraguerre and Clark, 2003). This topic was particularly investigated by a Canadian research group around T. F. Duffield. Aside from numerous own studies they conducted a meta-analysis analyzing monensin administration to lactating dairy cows in terms of metabolic, production, and health effects analyzing 59, 36, and 16 studies, respectively (Duffield et al., 2008a, 2008b, 2008c). The results were often heterogeneous, depending on dose, method, and duration of administration. However, in general, they concluded that monensin has the potential to decrease plasma NEFA and BHBA, increase MY, slightly decrease DMI, and it can help preventing ketosis, displaced abomasum as well as mastitis. However, the authors also detected an increased risk of dystocia and retained placenta in case of a prolonged treatment in the dry period (Duffield et al., 2008c). Mullins et al. (2012) observed decreased plasma BHBA and, additionally, slower hepatic TAG accumulation, without effects on MY and DMI, providing 400 mg/d of monensin to cows during whole transition period. Based on these results, they concluded that monensin must affect the metabolism in more ways than just in altering the propionate supply (Mullins et al., 2012).

Both the supplementation of glycerol and of propylene glycol is well established in commercial dairy farming. Recently, the use of a monensin product (Kexxtone®, Eli Lilly and Company Ltd, UK) for ketosis prevention has been authorized in Germany. However, this kind of use of monensin products is discussed very controversially as prophylactic treatment of livestock with antibiotics is generally rejected by the German consumers and the media (Liebrich, 2013; Tomic, 2013), who fear the abuse of pharmaceuticals like Kexxtone® for improving performance and increasing antibiotic resistance of pathogens (Cordts et al., 2013).

2.1.5 Other Management Factors

Aside from feeding strategies, there are management factors which can also affect the energy metabolism of transition dairy cows. These are not destined for maximizing DMI but rather for decreasing milk output during transition or shifting it to later stages of lactation. Several scientists investigated different dry period lengths and their effects on the cows' metabolism during the following lactation (compare reviews of Bachman and Schairer, 2003; Annen et al., 2004; Grummer and Rastani, 2004; van Knegsel et al., 2013; Santschi and Lefebvre, 2014). Traditionally, the dry period starts approximately 40 to 60 days prior to the expected parturition and mainly serves the purpose to regenerate the mammary tissue (Annen et al., 2004; Grummer and Rastani, 2004). When the focus is on maximizing the MY, a dry period of 50 to 70 days showed the best results (Grummer and Rastani, 2004; Ghavi Hossein-Zadeh and Mohit, 2013). However, this theory is increasingly questioned (van Knegsel et al., 2013). A shortened or even omitted dry period might be advantageous in terms of sparing fresh cows'

metabolism and easier dry cow management (Grummer and Rastani, 2004; van Knegsel et al., 2013). Some studies comparing traditional (control; approx. 60 d) to shortened (approx. 30 d) or no dry period observed higher MY for the control group in early lactation but no significant differences considering the performance of the whole lactation, as MY was shifted from early to late lactation (Pezeshki et al., 2008; Shoshani et al., 2014; van Knegsel et al., 2014b). Rastani et al. (2005) reported similar results for short and traditional dry periods but lower postpartal NEFA concentrations in continuously milked cows and concluded omitting the dry period could improve EB. An improved energy status was also observed in other studies with shortened and/or omitted dry periods (Pezeshki et al., 2008; Schlamberger et al., 2010; van Knegsel et al., 2014b; Chen et al., 2015; Chen et al., 2016). Others found no effects of a shortened dry period (Watters et al., 2008; Cermakova et al., 2014). There might be a potential to shift the load of milk performance from early lactation to late lactation but there might also be some additional individual factors and other management factors which have to be included in the determination of the dry period length (Bachman and Schairer, 2003; Steeneveld et al., 2014). Steeneveld et al. (2014), who investigated the association of cow characteristics and different dry period lengths, concluded that it might be possible to establish a management tool helping to determine dry period length according to the individual needs of the cow; for example, a shortened dry period for a multiparous cow which has a high MY at 12 weeks prepartum (Steeneveld et al., 2014). In summary, clear evidence for advantages of a shortened or omitted dry period are scarce but this also accounts for disadvantages. A recent meta-analysis showed reduced MY for shortened dry period with only a tendency to a lower incidence of ketosis and concludes that further research is required (van Knegsel et al., 2013).

Another method of reducing MY in early lactation and thus the metabolic load might be once daily milking for a certain time span after calving. Schlamberger et al. (2010) reported significantly reduced NEFA and BHBA concentrations, along with less BCS losses in cows which were milked once daily during the first four weeks of lactation. This led to the conclusion that once daily milking spares the early lactation metabolism, albeit at the expense of an approximately 20% reduced MY. Similar results of French studies were summarized by Rémond and Pomiès (2005), while O'Driscoll et al. (2012) additionally reported an improved immune function. A recent review summarizing different ways of once daily milking (short term or whole lactation) concluded that it might be a useful tool depending on the timing and the system to which it is applied (Stelwagen et al., 2013). Concerning udder health, an elevated somatic cell count (SCC) was reported in short as well as long term studies for once daily milking, but these observations were not necessarily related to increased incidence of mastitis (Stelwagen et al., 2013).

If management tools like once daily milking or shortened dry period really helped improving the cows' health and fertility and thus longevity, the costs for medical treatment and rearing of young stock were reduced and this might compensate the losses in MY per lactation. Additionally, the increased milk protein yields observed in cows milked once daily and cows with shortened or omitted dry period might also be of some economic relevance, as milk protein content is a component of milk pricing (Schlamberger et al., 2010; van Knegsel et al., 2013).

2.2 The Origin of Milk Fatty Acids

Milk is a highly complex product. Its composition has influence on the milk's taste and its physical properties. It is also of economic interest for farmers as the milk price per kg usually depends on milk protein and fat concentrations. Milk can also serve as a medium for health monitoring for dairy cows. Body fluids like urine or blood are often used to detect health problems in both human and animals, but milk is of particular interest in dairy cattle as it is an easily accessible medium. Milk can be used to discover health problems like mastitis by determining the SCC or ketosis by measuring acetone concentrations. Milk recording organizations give information to the farmer about the protein and energy supply of the cow using milk protein and urea concentrations or the fat to protein ratio. Yet, the informative value of these indicators is controversially discussed.

Of particular scientific interest is the composition of milk fat, primarily the FA profile. It is highly variable and depends on various genetic, metabolic, and nutritional factors which have been and still are subject of extensive research. Numerous studies dealt with the impact of diet composition and single feedstuffs, others examined animal factors which affect the milk FA profile. There have also been attempts to manipulate milk composition and milk fat composition in order to achieve beneficial effects for the processing industries or human health, such as increasing the *omega-3* (*n-3*) FA concentration (Davis, 2005). The main component of milk fat are TAG which account for approximately 98% of milk fat. Moreover, milk fat contains small amounts of diacylglycerols, monoacylglycerols, NEFA, and phospholipids. In addition, milk contains various compounds associated to milk fat such as sterols, fat-soluble vitamins, and β-carotenoids (MacGibbon and Taylor, 2006).

Bovine milk fat is considered one of the most complex natural lipids. The main reason for this is the enormous variety of milk FA, which can be combined to an even larger variety of TAG. Magidman et al. (1962) identified approximately 60 different FA, 27 of which were detected in concentrations lower than 0.1% (Herb et al., 1962). Forty years later, Jensen (2002) reported 416 individual milk FA, the vast majority at concentrations below 0.01%. Milk FA occur as

even-, odd- and/or branched-chain FA, in different chain lengths from 4 to 26 carbons, saturated or unsaturated containing zero to six double bonds at variable positions.

Milk FA arise from two main sources: from *de novo* synthesis in the mammary gland or from exogenous lipoproteins. *De novo* synthesized FA are SCFA containing 4 to 10 carbons and medium-chain fatty acids (MCFA) with 12 to 16 carbons (Bauman et al., 2011). However, only a part of the total C14:0 and C16:0 are synthesized *de novo*, while the rest originates from dietary lipids or from adipose tissue. The mammary gland sources long-chain fatty acids (LCFA) with chain lengths longer than 16 carbons from plasma NEFA or from plasma lipoproteins which are rich in TAG.

Table 1 displays the proportions of milk FA obtained from a meta-analysis of Moate et al. (2007). As these data arose from 28 publications with HF cows, which were in 103 ± 61 DIM, and 120 dietary treatments (including grazing experiments), the results can be regarded as a kind of reference milk FA profile. The most abundant FA in milk were palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0), and oleic acid (C18:1c9, OA) their proportions averaging 28, 10, 10, and 21 g/100 g of total milk FA, respectively. As the data in Table 1 are ordered by their weight, preformed FA seem to clearly dominate the FA profile. On molar basis, however, *de novo* synthesized and preformed FA contribute more or less in equal parts (Harvatine et al., 2009), but their contribution can change depending on several factors, for instance stage of lactation, energy status and diet. The ratio of SFA to UFA amounts to approximately 2:1. Odd-chain fatty acids (OCFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) occur in rather small amounts, but show a large variety of isomers.

2.2.1 Milk Fatty Acids of Dietary Origin

In ruminants dietary lipids usually do not reach the mammary gland without modification. In the rumen dietary lipids are hydrolyzed, followed by biohydrogenation of UFA and/or isomerization during this process. Additionally, FA are *de novo* synthesized by rumen microbes from carbohydrate precursors or dietary fat (Jenkins, 1993). Consequently, lipids which are absorbed in the intestine have a different FA profile than the dietary lipids taken up. Basically, lipids which leave the rumen contain considerably more stearic acid than the diet and less C18 UFA which represent the majority of FA in feedstuffs (Harfoot and Hazlewood, 1997). Linoleic acid (LA, C18:2c9,c12) and α-linolenic acid (ALA, C18:3c9,c12,c15), are the most important FA in forages, while concentrates mainly contain LA and OA (Harfoot and Hazlewood, 1997).

The ruminal fermentation of carbohydrates is the main source of volatile FA (mainly acetate, propionate, and butyrate), which can serve as precursor for FA *de novo* synthesis of rumen

microbes, the mammary gland, and other tissues. The most important precursor is acetate, followed by butyrate, which both mainly arise from cellulose degradation. Propionate, however, predominantly originates from starch and sugar degradation and is far more important as substrate for gluconeogenesis (Christie, 1981; Dijkstra et al., 2005).

Table 1 Concentrations of individual milk fatty acids (FA, g/100g of total FA). Adapted and modified from Moate et al. (2007)

FA	Mean	SD	FA	Mean	SD
C4:0	3.13	0.68	C18:1c9	20.50	5.35
C6:0	1.94	0.52	C18:2c9,c12	3.13	2.11
C8:0	1.17	0.35	C18:2c9,t11	1.02	0.6
C10:0	2.48	0.73	C18:2t10,c12	0.04	0.03
C12:0	2.99	0.85	C18:2c11,t13	0.04	0.03
C14:0	10.38	1.71	Other CLA	0.15	0.14
14:1c9	1.08	0.36	C18:3	0.59	0.36
C15:0	1.05	0.33	C20:0	0.15	0.06
C16:0	28.51	4.98	C20:5	0.10	0.11
C16:1c9	1.73	0.63	C22:6	0.07	0.07
C17	0.73	0.35	Others	7.51	5.62
C18:0	10.51	3.59	∑CLA	1.03	0.66
C18:1t6–t8	0.46	0.21	∑18:1 *trans*	4.25	2.63
C18:1t9	0.44	0.20	∑*de novo*	23.26	4.24
C18:1t10	1.31	1.52	∑C16	30.09	5.27
C18:1t11	3.33	2.18	∑preformed	46.65	7.58
C18:1t12	0.65	0.36			

These data were obtained in a meta-analysis using 28 publications in which 120 dietary treatments were investigated.
c = *cis*; t = *trans*; CLA = conjugated linoleic acid; ∑18:1 *trans* = sum of all C18:1trans isomers; ∑de novo = sum of C4:0 to C15:0; ∑C16 = sum of C16:0 and C16:1c9; ∑preformed = sum of FA≥C17:0

2.2.1.1 Hydrolysis and Biohydrogenation in the Rumen

The major sources of FA in forages are galactolipids, phospholipids, and sulpholipids which are mainly part of the chloroplast membrane, while concentrates predominantly contain TAG (Harfoot and Hazlewood, 1997; Jenkins et al., 2008). These lipids are rapidly hydrolyzed by microbial lipolytic enzymes in the rumen. Thus, the ester bonds are solved and free FA are released. Lipolysis is an essential step prior to biohydrogenation of UFA, as a free carboxyl group is required (Jenkins, 1993). Linoleic acid, for example, is then transformed to C18:2c9,t11 by an isomerase which catalyzes the conversion of the *cis*-12 to a *trans*-11 double bond. Following this step, the biohydrogenation itself takes place: a reductase hydrogenates the *cis*-9 double bond, forming the monoene vaccenic acid (C18:1t11), which is then further reduced to stearic acid. Different types of bacteria which produce enzymes for different steps of biohydrogenation were identified (Harfoot and Hazlewood, 1997). They are divided in two

groups. Group A is suspected to not be able to hydrogenate C18 MUFA, they mainly hydrogenate LA and ALA to *trans*-C18:1 FA, while species of group B can do both, as shown in Figure 2 (Bauman and Lock, 2006b).

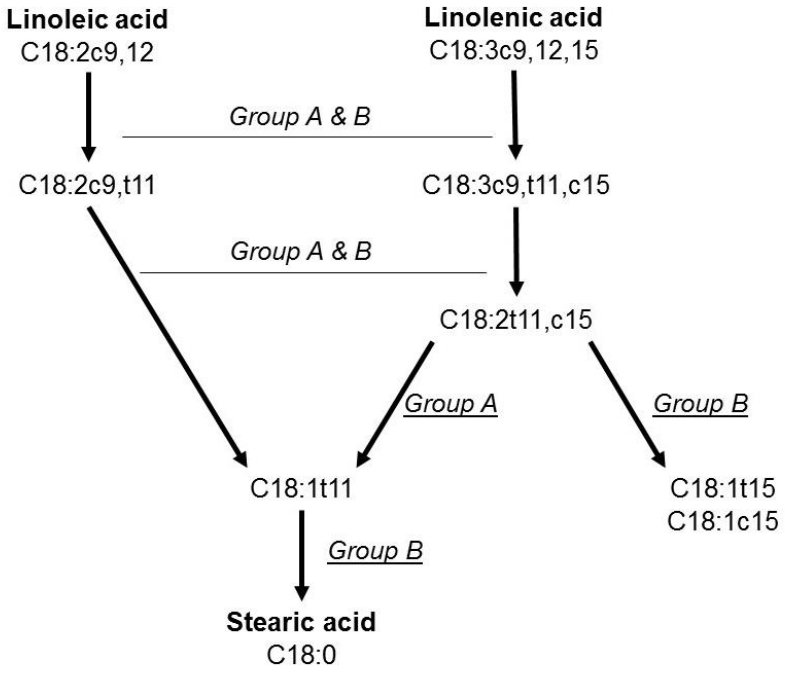

Figure 2 Ruminal biohydrogenation of linoleic and linolenic acid. Adapted and modified from Bauman et al. (2001), based on Kemp and Lander (1984).

Oleic acid is also normally first transformed to a *trans*-octadecenoic acid and then hydrogenated to stearic acid (Mosley et al., 2002; Vossenberg and Joblin, 2003). Some rumen fungi were found to be able to hydrogenate LA to vaccenic acid but it takes them much longer to complete this process than rumen microbes (Nam and Garnsworthy, 2007). The displayed processes of isomerization and biohydrogenation in Figure 2 are not unique for individual FA. A number of other pathways of isomerization exists from which various different isomers can emanate, which lead to diverse C18 monoenes as dead end products that cannot be further hydrogenated (Bauman et al., 2001; Jenkins et al., 2008; Shingfield et al., 2010). Additionally, intermediates can escape further biohydrogenation and reach the intestine, for instance vaccenic or rumenic acid (C18:2c9,t11), a CLA, and numerous other CLA and *trans* isomers (Jenkins et al., 2008; Lee and Jenkins, 2011).

Ruminal biohydrogenation is extensive and initiated very fast, as PUFA are suspected to have toxic effects on the rumen microbial system (Jenkins, 1993; Maia et al., 2007). However, the extent and products of isomerization and biohydrogenation of free dietary FA in the rumen is highly variable and depends on diet composition, which can induce shifts in the microbial

population, on particle size, and the rate of passage of digesta from the rumen (Jenkins, 1993; Harfoot and Hazlewood, 1997; Bauman et al., 2001). Linolenic acid is nearly completely hydrogenated (on average 92%), while the biohydrogenation of LA occurs to an extent of 70 to 95% depending, inter alia, on the concentrate level in the diet (see section 2.3.2.3; Doreau and Ferlay, 1994).

2.2.1.2 Microbial Fatty Acid Synthesis

Rumen microbes can directly incorporate dietary FA into their cells but especially bacteria also synthesize FA *de novo* (Jenkins, 1993). Mainly, palmitic and stearic acids are produced, but also a number of OCFA and branched-chain fatty acids (BCFA) in milk are known to be predominantly of microbial origin. The general process of FA *de novo* synthesis is described in section 2.2.3.

There are two types of fatty acid synthase which differ in their affinity to the respective primers (Kaneda, 1991). Odd-chain FA mainly originate from microbial *de novo* synthesis using propionyl-CoA (C3) or valeryl-CoA (C5) instead of acetyl-CoA to gain C15:0 and C17:0 and are synthesized by straight-chain fatty acid synthase (Vlaeminck et al., 2006a). Whether acetyl-CoA or propionyl-CoA are used as primers for straight-chain FA synthesis seems to depend on their respective availability (Fulco, 1983). On the other hand, BCFA are generated by branched-chain fatty acid synthase for instance from isobutyryl-CoA, isovaleryl-CoA or 2-methylbutyryl-CoA primers, which result in three groups of BCFA: C14:0*iso* and C16:*iso*, C15:0*iso* and C17:0*iso* or C15:0*anteiso* and C17:0*anteiso*, respectively (Kaneda, 1977, 1991; Jenkins, 1993; Vlaeminck et al., 2006a). Shifts in the profile of OCFA and BCFA can occur due to the affinity of the primers to the respective fatty acid synthase, which in turn depends on the composition of the bacterial population as some fatty acid synthases are specific for individual bacterial species. Furthermore, the availability of primers and malonyl-CoA for chain elongation can vary according to physiological and cultural conditions and can thus influence the BCFA profile (Kaneda, 1977). But these factors play an subordinate role in the variation of BCFA and OCFA in comparison to the abundance of the types of fatty acid synthase themselves, which in turn depend on the bacterial populations in the rumen (Vlaeminck et al., 2006a).

Up to 20% of the microbial *de novo* synthesized FA are MUFA, C16:1, and C18:1, which are synthesized via the anaerobic pathway (Fulco, 1983). Within this pathway, the C10:0 intermediate is formed to β-hydroxy-C10:0, then dehydrated to C10:1c3. As no further reduction is possible, the double bond is maintained which leads to the formation of C16:1c9 or C18:1c11 after chain elongation using malonyl-CoA (Fulco, 1983).

When the side-chain phytol is cleaved off chlorophyll it can be hydrogenated and then oxidized to the multi-branched FA phytanic acid by rumen microbes. This FA and its derivative pristanic acid, which is formed via α-oxidation in hepatic peroxisomes, occur in milk fat in small amounts, approximately 0.06 and 0.36 g/100 g of milk fat (Singh, 1997; Parodi, 2006; Vlaeminck et al., 2006a). The concentrations of these FA in milk and milk products depend on the proportions of grass based feedstuffs in the diet which might allow differentiation of organic and conventional products. Organic dairy products were found to contain 50% more phytanic and 30% more pristanic acid than conventional ones (Vetter and Schröder, 2010).

Catabolism of FA by rumen microbes is considered to be very low (Jenkins, 1993). Nevertheless, in a meta-analysis comparing duodenal dietary lipid flow to lipid uptake across 15 studies Jenkins (1993) calculated a negative ruminal lipid balance of 8 g per 100 g lipid uptake. Some studies even observed a loss of more than 30 g (Bauchart et al., 1987; Wu et al., 1991). This may be a consequence of dietary fat supplementation, as bacteria seem to reduce the *de novo* synthesis in favor of direct FA incorporation when external FA supply increases (Jenkins, 1993; Doreau and Ferlay, 1994). However, to which extent microbial lipids account for total FA leaving the rumen is highly variable, since the microbial ecosystem strongly interacts with the diet composition (Noble, 1981).

2.2.2 Transport of Preformed Fatty Acids and Uptake by the Mammary Gland

Most free FA up to C12 in the digesta are already absorbed by the rumen epithelium and this way attain the bloodstream (Doreau and Ferlay, 1994). Lipids in the duodenal digesta are mainly composed of free saturated MCFA and LCFA (predominantly stearic acid) and some microbial and biliary phospholipids but as the case may be also TAG of protected fats (Bauchart, 1993; see section 2.3.2.4). Phospholipids and TAG are hydrolyzed to NEFA and β-monoacylglycrols by the pancreatic lipase/phospholipase system, which then form micelles together with biliary lysolecithin. The hydrophobic parts of the molecules are arranged in the center of these micelles while the hydrophilic parts are located at the exterior. This arrangement allows solubilization of the FA with the aqueous intestinal liquid which is prerequisite for FA absorption (Doreau and Chilliard, 1997; Bauman and Lock, 2006a).

Generally, the intestinal absorption of FA is more efficient in ruminants than in non-ruminants (Noble, 1981). According to a meta-analysis comprising 20 trials presented by Lock et al. (2006), the mean digestibility of total FA is approximately 74%, ranging from 58 to 86%. These results are similar to the information given by Doreau and Ferlay (1994).

Once absorbed by the intestinal epithelium, the FA are resynthesized to TAG and packaged into lipoproteins. These lipoproteins are mostly VLDL and chylomicra: a transport form of

dietary FA from the intestine and the liver (Kronfeld, 1982; Barber et al., 1997; see section 2.1.1.2). They are released into the lymph system, which explains the near absence of chylomicra in the portal vein blood, and subsequently delivered into the blood (Bauchart, 1993; Doreau and Ferlay, 1994). Once in the plasma, the lipoproteins are available for the peripheral tissues.

As lipoproteins are too large to enter their respective target cells (for instance myocytes, adipocytes or mammary epithelial cells), their TAG are previously hydrolyzed extracellularly by lipoprotein lipase located at the luminal surface of vascular endothelial cells (Barber et al., 1997; Clegg et al., 2001). However, the mammary uptake of this split off FA is not very efficient. An early study of Mendelson and Scow (1972) demonstrated in rats that 60% of FA from lipoproteins hydrolyzed by lipoprotein lipase were taken up by mammary epithelial cells, while the residual 40% returned into blood circulation. This is one reason why usually no net uptake of plasma NEFA occurs in mid-lactation: on the one hand, plasma NEFA are taken up by the mammary gland, on the other hand, FA of hydrolyzed lipoproteins are released into the blood. Net NEFA uptake can only be observed in times of high plasma NEFA concentrations, hence NEB, early after parturition or in subclinical ketosis (Miller et al., 1991; Nielsen and Jakobsen, 1994).

2.2.3 De novo Synthesis of Fatty Acids in the Mammary Gland

Fatty acid synthesis requires two types of precursors: a carbon source and reducing equivalents in the form of NADPH. In non-ruminants, glucose serves as a precursor for both but the ruminant mammary gland uses acetate and BHBA as carbon sources as well as glucose and acetate to generate NADPH (Palmquist, 2006). Acetate arises mainly from ruminal fermentation but also from β-oxidation, BHBA either from ruminal butyrate converted in the rumen epithelium or from β-oxidation. Glucose is mainly transported into the mammary epithelial cells by the protein GLUT1 (glucose transporter 1) which is independent from blood insulin levels (Zhao et al., 1993). NADPH is mainly produced from dehydrogenation of isocitrate to α-ketoglutarate or, to a lesser extent, in the course of glycolysis via the pentose phosphate pathway.

The first step of the *de novo* synthesis is the activation of acetate to acetyl-CoA by acetyl-CoA-synthase in the cytosol of the mammary epithelial cells (Moore and Christie, 1979). Some acetyl-CoA is then turned into malonyl-CoA promoted by acetyl-CoA carboxylase. The following steps of the actual FA synthesis are catalyzed by a single multifunctional polypeptide named fatty acid synthase (Figure 3). It contains seven enzymes to which the intermediates are transferred subsequently (Smith, 1994). Acetyl-CoA serves as a primer which is transferred

from acetyl-/malonyltransferase to the acyl carrier protein (ACP) and then to β-ketoacyl synthase. Malonyl-CoA, which is used as substrate for chain elongation, is processed via the same pathway. *Beta*-ketoacyl synthase catalyzes the condensation of the primer and malonyl-CoA. Three more enzymes (β-ketoacyl reductase, dehydrase, and enoyl reductase) are involved in the formation of butyryl-S-ACP, to which then another malonyl-CoA can be attached by repeat of the preceding reactions. This cycle is performed until the FA is released by thioesterase, which happens at a chain length of 12 to 16 carbons. To achieve C16:0, acetyl-CoA has to be subsequently combined to 7 malonyl-CoA, using 14 NADPH and 14 H^+ (Smith et al., 2003). A detailed description of the whole process was provided by Smith et al. (2003). BHBA is also activated to its CoA derivative β-hydroxybutyryl-CoA, which can be used by acyltransferase. It exclusively serves as a primer for FA synthesis and as about half of the *de novo* synthesized FA are derived from a BHBA primer, it accounts for approximately 8–9% of total carbons in milk FA, while acetate contributes approximately 42% (Palmquist et al., 1969; Smith et al., 1974). Mammary fatty acid synthase can also produce linear OCFA (C15:0, C17:0) using propionyl-CoA as primer. However, its contribution is low and the vast majority of OCFA originates from the microbial *de novo* synthesis in the rumen (Vlaeminck et al., 2006a).

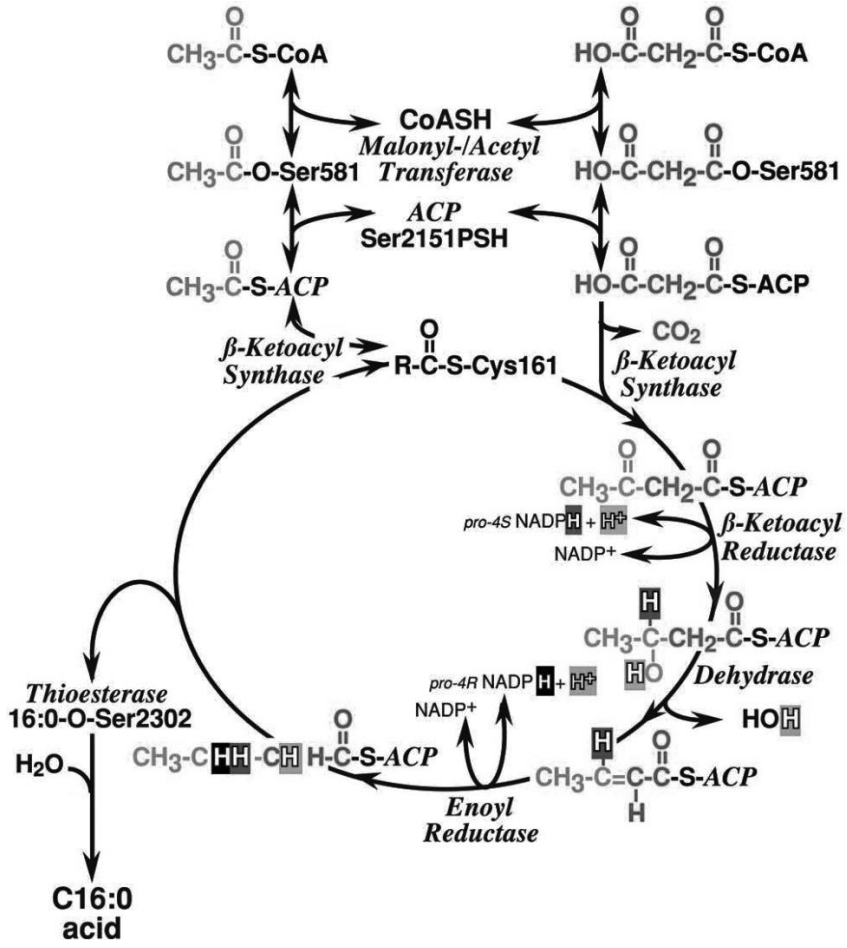

Figure 3 *De novo* synthesis of FA catalyzed by fatty acid synthase (adapted from Smith et al., 2003; reproduced with permission of Elsevier)

The presence of considerable amounts of SCFA in milk fat is typical for ruminants (MacGibbon and Taylor, 2006). They originate from incomplete *de novo* synthesis of MCFA, when intermediates escape during the transfer from acetyl-/malonyltransferase to β-ketoacyl synthase (Palmquist, 2006). The conversion of C16:0 to C18:0 by chain elongation is not possible in the mammary gland – contrary to other ruminant tissues (Palmquist, 2006).

2.2.4 Stearoyl-CoA Desaturase

Exclusively SFA are *de novo* synthesized in ruminant tissues and the level of saturation of dietary FA is also high due to ruminal biohydrogenation. However, as a high degree of saturation results in a higher melting point of fats, a system of desaturation exists to contribute to an increased fluidity of (milk) fat (Chilliard et al., 2000b; Chilliard et al., 2007). Stearoyl-CoA desaturase (SCD) is an enzyme located predominantly in the endoplasmic reticulum of secretory cells in the lactating mammary gland and in adipose tissue but also to some extent in intestinal enterocytes (Palmquist, 2006). It catalyzes the formation of a *cis*-9 double bond, which is why SCD is also named *delta*-9 desaturase. SCD mainly uses stearic and palmitic acid for substrate but also dehydrogenates small amounts of other SFA, such as C10:0, C12:0, C14:0, C15:0, and presumably C17:0 (Bickerstaffe and Annison, 1970; Vlaeminck et al., 2006a; Bernard et al., 2013). The desaturation of stearic acid is the most important reaction, with approximately 50 to 55% of available stearic acid being transformed to OA in the mammary gland (Enjalbert et al., 1998; Mosley and McGuire, 2007). The amount of total milk OA emerging from SCD activity ranges from 43 to 78% (Griinari et al., 2000; Corl et al., 2001; Mosley and McGuire, 2007), depending on the stearic acid supply and the direct uptake of OA (Noble et al., 1969; Jensen et al., 1991).

Trans-C18:1 FA emerging from ruminal biohydrogenation can serve as a substrate for SCD as well. For instance, the conversion of C18:1t11, C18:1t7 or C18:1t12 leads to C18:2c9t11 CLA, which is the major CLA in milk fat, C18:2t7c9 CLA, and C18:2c9t12, respectively (Griinari et al., 2000; Corl et al., 2002; Shingfield et al., 2010). Griinari et al. (2000) suspected that relatively high amounts of CLA can be synthesized endogenously from vaccenic acid, which was confirmed shortly afterwards by Corl et al. (2001), who estimated that even 78% of milk C18:2c9t11 are of endogenous origin.

2.3 Factors Affecting the Composition of Milk Fat

2.3.1 Animal Factors on Milk Fatty Acid Profile

There are some animal factors which can influence the FA composition of milk (for comprehensive information see reviews of Palmquist et al., 1993; and Samková et al., 2012). Genetic aspects do impact milk FA composition in dairy cows – many studies were conducted to examine differences between dairy breeds, genetic lines within breeds, and differences on individual cow level (Stull and Brown, 1964; DePeters et al., 1995; Kelsey et al., 2003; Secchiari et al., 2003; Soyeurt et al., 2006b; Palladino et al., 2010; Weisbjerg et al., 2013).

Stull and Brown (1964) compared the milk FA profile of Holstein, Jersey, and Guernsey cows. They reported significantly lower concentrations of C10:0 and C12:0 in Holstein Frisian milk fat, while C16:0, C16:1, and C18:1 occurred in higher concentrations compared with Jersey and Guernsey cows. Later, other studies showed that breeds having a high milk fat content, for instance Jersey, also have higher proportions of MCFA and SFA than others (White et al., 2001; Samková et al., 2012; Marchitelli et al., 2013). Some authors ascribe inter-breed differences in milk FA profile to variations in SCD activity (Mele et al., 2007; Schennink et al., 2008). Yet, the results are often ambiguous, as different feeding strategies or considerable differences in sample size complicate direct comparison of studies investigating the same breeds (Samková et al., 2012).

Genetic aspects of milk FA proportions were investigated intensely during the last decades achieving low to moderate values of heritability of individual FA (Samková et al., 2012). Low heritabilities (up to 0.2) were estimated for LCFA, which can be explained by their dietary origin, while *de novo* synthesized FA showed higher values from 0.3 to 0.5 (Stoop et al., 2008; Bastin et al., 2011). Kay et al. (2005) demonstrated in two genetic lines of Holstein cows that the concentrations of most milk FA were not affected by prolonged selection for high or low merit. Then again genetic strains of Holsteins from New Zealand showed greater proportions of C8:0 to C12:0 and CLA and lower concentrations of OA compared with North American Holsteins (Meier et al., 2013). Additionally, there was great effort in identifying lipogenic genes and exploring their polymorphisms (Bernard et al., 2008; Samková et al., 2012; Mach et al., 2013; Marchitelli et al., 2013; Nafikov et al., 2014; Pegolo et al., 2015; Pegolo et al., 2016).

Parity as a factor of variation in milk FA is scarcely examined. In some studies no effect of parity on milk FA profile was detected (Secchiari et al., 2003; Kgwatalala et al., 2009), while a majority showed an effect, even if small (Kelsey et al., 2003; Craninx et al., 2008; Soyeurt et al., 2008). Most notably there are differences between primiparous and multiparous cows. Recently, Bilal et al. (2014) reported that milk of primiparous cows contained higher

concentrations of OA, ALA, vaccenic acid and CLA and lower concentrations of C12:0 to C16:0 than milk of multiparous cows. These findings are largely consistent with the results of Craninx et al. (2008). Lower metabolical activity of the mammary gland and lower fatty acid synthase expression in primiparous cows may explain the decreased proportions of *de novo* synthesized FA (Miller et al., 2006).

2.3.1.1 Stage of Lactation and Negative Energy Balance

In 1966, observing four Holstein cows during their whole lactation, Stull et al. hypothesized that due to the high initial and then declining concentrations of palmitic acid, stearic acid and OA in milk, body fat mobilization might play a role in the milk FA profile changes during lactation. This was confirmed by a number of studies since that time. As mentioned before albumin bound plasma NEFA can also be taken up by the mammary gland and incorporated into milk fat (Kronfeld, 1982). They arise from hydrolyzed TAG of the adipose tissue, which are released into the blood (see section 2.1.1.2). In cows sufficiently supplied with energy plasma NEFA from adipose tissue account for 5 to 8% of milk FA, but as the extent of mammary NEFA uptake depends on plasma NEFA concentrations, this proportion increases with increasing body fat mobilization (Miller et al., 1991; Bauman and Griinari, 2001; Chilliard et al., 2003). Thus, milk fat can contain up to 40% FA derived from the plasma NEFA pool when the cow experiences NEB early postpartum (Bell, 1995).

Consequently, it might be suspected that the plasma NEFA profile largely corresponds to the FA composition of the adipose tissue in times of lipomobilization as assumed by Loften et al. (2014). And indeed, both contain the same predominant FA, as there are palmitic acid, stearic acid, and OA (Rukkwamsuk et al., 2000; Douglas et al., 2004; Contreras et al., 2010). In subcutaneous adipose tissue of dairy cows the concentrations of these FA range between 26–30% for palmitic acid, 9–17% for stearic acid, and 41–50% for OA, whereby the high concentration of OA results from the SCD activity in adipose tissue (Rukkwamsuk et al., 2000; Douglas et al., 2007; Zachut et al., 2010). In most studies the plasma NEFA composition is not analyzed separately but the composition of total plasma lipids. Rukkwamsuk et al. (2000) showed in periparturient cows, which were overfed during the dry period and were intensely mobilizing body fat postpartum, that concomitant to the postpartal increase of NEFA concentrations in blood (up to 546%) the concentrations of palmitic acid, stearic acid, and OA in total serum FA rose disproportionally by 435, 704, and 823%, respectively. These results were generally confirmed by Zachut et al. (2010). However Contreras et al. (2010) showed marked differences in the concentrations of stearic acid (40% prepartum, 49% at 30 DIM) and OA (10% prepartum, 3% at 30 DIM) in plasma NEFA compared with the values for adipose tissue given before. There are several hypotheses which might explain these differences. First, different fat depots have different FA profiles, as shown by de Smet et al. (2004), being more

saturated the deeper they are located in the animals' interior. Yet, Hostens et al. (2012) reported still slightly higher concentrations of OA than stearic acid in the abdominal fat of cows (31 vs. 26%, respectively), whereby it is to take into account that all probed animals suffered from left displaced abomasum. In this trial the plasma NEFA profile showed 20 and 26% of stearic acid and OA, respectively. Another explanation might be that individual FA are selectively released by the adipose tissue as shown in humans (Raclot, 2003). However, at least during the first three weeks of lactation, there are no striking changes in the adipose tissue FA profile (Rukkwamsuk et al., 2000). It is also possible that OA from plasma NEFA is preferentially taken up by other tissues, in this case particularly by the mammary gland or the liver, which might lead to relatively lower plasma levels.

Despite this lack of clarity it is common knowledge today that the milk fat composition changes with stage of lactation and in times of NEB and concomitant lipomobilization. In early lactation the high uptake of LCFA from plasma NEFA causes an inhibition of the *de novo* synthesis in the mammary gland (Palmquist et al., 1993). As a consequence the proportion of FA from C6 to C15 is low in the beginning, yet increasing with progressing lactation and declining NEB, while main LCFA act reversely (Palmquist et al., 1993; Kay et al., 2005; Garnsworthy et al., 2006; Gross et al., 2011a; Ducháček et al., 2012; Nogalski et al., 2012; Bilal et al., 2014). Milk butyric acid (C4:0) acts differently than other SCFA: its concentrations are increased in early lactation as its utilization as a primer is diminished due to the inhibition of *de novo* synthesis (Palmquist et al., 1993; Bilal et al., 2014). Gross et al. (2011a) showed that the milk FA composition changes similarly during a deliberately induced NEB (approx. -65 MJ NEL/d) in mid-lactation compared with the natural one (-45 MJ NEL/d in week 1 postpartum) in early lactation (Figure 4). Yet, in spite of a greater NEB these changes were less pronounced and showed a trend to recover while NEB was still maintained (Gross et al., 2011a). Kay et al. (2005) reported increasing levels of *trans* C18:1, LA, and rumenic acid during the first 16 weeks of lactation, which was confirmed by Gross et al. (2011a) and Bilal et al. (2014) for vaccenic and rumenic acid.

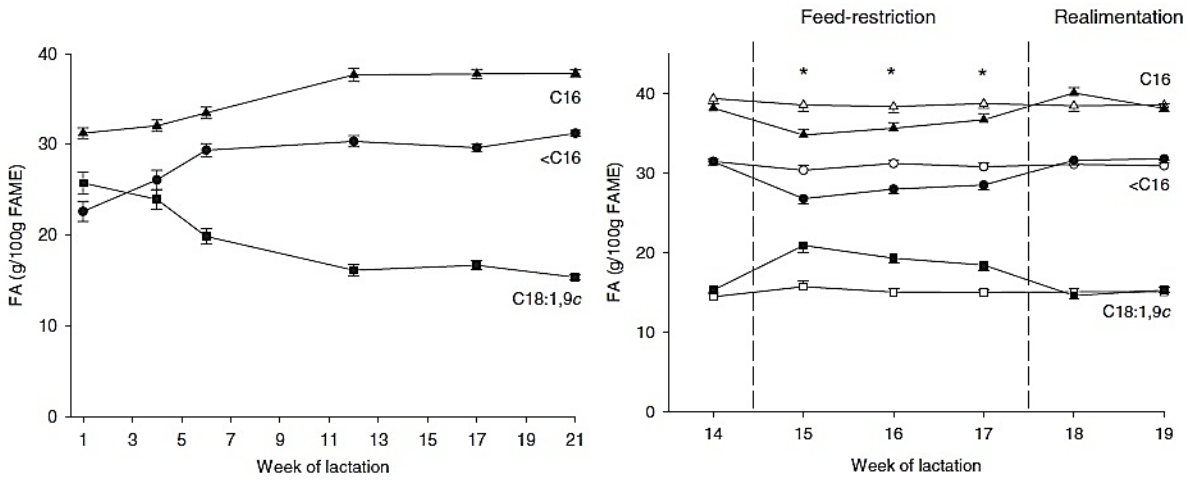

Figure 4 Proportions (g/100g FA methyl esters, FAME) of milk C16:0 (triangles), FA<C16 (circles), and FA>C16 (squares) during the first 21 weeks of lactation (left) and induced NEB (right) during mid-lactation. Solid symbols on the right represent feed restricted cows, while empty symbols on the right show control cows, stars indicate significant differences ($p<0.05$). Adapted from Gross et al. (2011a), reproduced with permission of Cambridge University Press.

2.3.2 Nutritional Effects on Milk Fatty Acid Composition

As previously stated, a great proportion of milk FA composition depends on diet composition. Feedstuffs, supplements, and their nutrient/fat composition do not only directly contribute to milk fat, but can also influence the mammary *de novo* synthesis and thus change the milk FA profile. Nutrition as a source of variation in milk fat composition was subject of extensive research during the last decades, which led to a number of reviews dealing with this topic (Palmquist et al., 1993; Ashes et al., 1997; Walker et al., 2004; Palmquist, 2006; Chilliard et al., 2007). In addition, dairy products are highly important foodstuffs in human nutrition on the one hand, but on the other hand suffer from a rather bad reputation because of their relatively high amounts of hypercholesterolemic FA, i.e. SFA, especially myristic and palmitic acids (Demeyer and Doreau, 1999; Jensen, 2002; Lock and Bauman, 2004). Consequently, the dairy industry longs for an increase of the proportions of FA which are considered beneficial for human health (Jensen, 2002). These include UFA in general and especially *n-3* FA (e.g. C18:3c9,c12,c15; C20:5c5,c8,c11,c14,c17; C22:6c4,c7,c10,c13,c16,1c9) and CLA, which are of particular interest due to their anticarcinogenic, anti-atherogenic, anti-obesity, immunomodulating, and other health improving effects which were shown in animal models (Belury, 2002). Consequently, researching the potential to change cows' milk FA composition through nutrition became not only a scientific desire, but also an interest of human nutrition, and thus of economic relevance.

In the following, only some examples of dietary strategies for manipulation of milk fat composition are presented, for detailed information the reader is referred to the aforementioned review articles.

2.3.2.1 Milk Fat Depression

As mentioned in section 2.1.4.1, there are dietary conditions which induce milk fat depression, especially when the diets contain high proportions of PUFA. When milk fat depression occurs, the milk FA profile changes significantly, showing increased proportions of preformed FA at the expense of *de novo* synthesized ones (Bauman and Griinari, 2003; Shingfield et al., 2010). Bauman and Griinari (2003) reviewed and discussed several theories and respective investigations concerning the origin of this phenomenon and concluded that the *de novo* synthesis in the mammary gland must be directly inhibited by *trans* FA, originating from ruminal biohydrogenation of dietary UFA. The main actors are suspected to be C18:1t10 and C18:1t10,c12 (Bauman and Griinari, 2003).

2.3.2.2 Effect of Forages

Between 20 and 100% of the energy requirements of cows are covered by forages, depending on farming/feeding system (Halmemies-Beauchet-Filleau et al., 2013b). Despite their rather low FA concentrations (2–5% DM) their importance as a source of FA is indisputable (Harfoot and Hazlewood, 1997; Kalač and Samková, 2010). Fresh or preserved, forages mainly contain ALA, LA, and palmitic acid in varying concentrations depending on botanical composition, season, climate, maturity level, fertilization, and preservation method. These factors and their potential to influence milk FA profile have been extensively reviewed (Chilliard et al., 2001b; Dewhurst et al., 2006; Elgersma et al., 2006; Kalač and Samková, 2010). Furthermore forages are the most important source of dietary fiber, which is inevitable for the rumen function and provides acetate in the course of ruminal fermentation which in turn is the main precursor for mammary FA synthesis (Ashes et al., 1997).

In general, highest levels of FA are found in young plants of fresh forage, decreasing with approaching summer (and thus preceding stage of vegetation) and in some cases recovering in autumn (Dewhurst et al., 2001). ALA is the major FA in fresh forage (>50% of total FA), followed by palmitic (~16%) and LA (~14%; Kalač and Samková, 2010). It is well documented that grazing cows' milk contains higher proportions of UFA and lower proportions of MCFA than milk of animals fed diets containing conserved forages. Particularly ALA, vaccenic acid, and CLA have elevated concentrations in milk from pasture (Chilliard et al., 2001b; Dewhurst et al., 2006; Ferlay et al., 2006; La Terra et al., 2010). According to Leiber et al. (2005) there

is no significant difference in milk FA composition if cows are either grazing or barn fed with fresh grass, so there will be no strict determination in the further course of this text.

Couvreur et al. (2006) showed a positive linear relationship between increasing intake of fresh grass (0–100%) and milk ALA, vaccenic, and rumenic acid concentrations, a negative one for concentrations of C10–C16. Eight fistulated Holstein cows were applied to four diets in which corn silage was replaced by fresh grass by 0, 30, 60, and 100% according to a Youden square design. The cows were supplemented with 3 kg soybean/cereal concentrates per day, containing decreasing proportions of soybean with increasing proportions of grass in the diet. Total milk FA of cows fed 0% fresh grass contained 0.22, 0.85, and 0.48 g/100 g of ALA, vaccenic, and rumenic acid, respectively, increasing to 0.70, 4.70, and 1.65 g/100 g FA for cows fed 100% fresh grass. The ALA concentrations in milk rise because more ALA can escape the ruminal biohydrogenation due to the elevated supply (Chilliard et al., 2007). A high supply of substrate for ruminal biohydrogenation also leads to an increased transfer of intermediates (vaccenic acid above all) to the mammary gland (Chilliard et al., 2007). However, as rumenic acid is mainly an intermediate of the biohydrogenation of LA, not ALA, elevated CLA concentrations in milk of cows on pasture are rather attributed to a higher desaturase activity in the mammary gland than to an increased formation in the rumen (Lahlou et al., 2014). Mohammed et al. (2009) showed that 95% of the variation in milk rumenic acid concentrations is ascribed to plasma vaccenic acid concentrations. Despite the low proportions of OA in grass, it is often observed that cows on pasture also show increased OA concentrations in milk (Ferlay et al., 2006; La Terra et al., 2010; Lahlou et al., 2014). This can be referred to high mammary C18:0 absorption as a result of the biohydrogenation of ALA and mammary desaturase activity (Schroeder et al., 2004).

If the concentrations of certain milk FA increase others inevitably decrease. Couvreur et al. (2006) showed that mainly milk palmitic acid, and to a lower extent myristic acid, decrease with increasing uptake of fresh grass, which is in consistence with reports of other authors (Chilliard et al., 2001b; Lahlou et al., 2014). Effects on SCFA, like C6–C8, and for stearic acid are inconsistent (Chilliard et al., 2007).

Milk from alpine pastures is often observed to be richer in OA and ALA than from lowland pastures, even if the FA composition of alpine pastures shows no higher contents of these FA (Chilliard et al., 2007). Elevated OA might result from a higher mobilization of body reserves due to alpine conditions, while the ruminal biohydrogenation might be compromised by either energy deficiency or secondary plant ingredients of alpine pastures and thus lead to increased milk ALA contents, as hypothesized by Leiber et al. (2005). Dewhurst et al. (2006) reported lower C12–C16 contents in milk from cows on alpine compared with lowland pasture.

As shown above the consumption of fresh forages affects milk FA profile in a different way than conserved forages. But there are also differences within the substrates of conservation (grass/corn/legumes) and their conservation method. Hay has a different FA profile than grass silages. Oxidative losses (esp. PUFA) during the process of wilting and drying and losses of leaf material due to disintegration cause lower total FA content and lower ALA concentrations in hay (Dewhurst et al., 2006). Short-time wilting for ensiling does not have such a significant effect. Glasser et al. (2013) conducted a meta-analysis concerning FA contents and profiles of forages. They reported average total FA contents of 20.1, 17.8, 12.9, and 7.7 g/kg DM and ALA contents of 52.6, 49.8, 47.1 and 23.6 g/100 g FA for fresh multi species forages, silage, hay, and low quality hay, which got damp during production, respectively. Comparable results were reported by Halmemies-Beauchet-Filleau et al. (2013a) and Shingfield et al. (2005), who both compared hay and various silages (differing in supplementation of ensiling agents) produced from the same swards. Despite the markedly lower ALA contents in hay, milk from hay fed cows is reported to contain higher LA and ALA proportions (1.21 and 0.50 g/100 g FA) than from grass silage fed cows (0.96 and 0.35 g/100 g FA; Shingfield et al., 2005). Apparent transfer rates from the diet into milk were 29 and 17% from hay and 15 and 3% from silage for LA and ALA, respectively (Shingfield et al., 2005). Yet, these findings were not confirmed by Halmemies-Beauchet-Filleau et al. (2013b), who did neither detect significant differences in milk ALA and LA nor in total PUFA concentrations when comparing hay and grass silage feeding.

The comparison of milk FA profiles of cows fed grass silages preserved with or without ensiling agents in the two aforementioned studies showed minor effects. However, Shingfield et al. (2005) reported significantly higher proportions of milk ALA, C18:1*trans*, and CLA when cows were fed silages treated with an inoculant enzyme preparation instead of formic acid.

A comparison of diets containing either grass silage, clover silage, or alfalfa silage as forage components showed increasing contents of LA and ALA in milk for legume silages, while clover silages had a greater impact (+27% and +97%) than alfalfa (+13% and +26%) compared with grass silage (Dewhurst et al., 2003). Clover silages led to a decrease in milk palmitic acid. Furthermore, Dewhurst et al. (2003) observed increased MY (up to +6 kg for white clover) when legume silage was fed compared with grass silage, due to increased DMI. These results are consistent with the results of Steinshamn (2010) who reviewed 31 studies from 1983 to 2009 that compared the use of grass silage and different legume silages or legume silages among themselves in diets (including Dewhurst et al., 2003). Recently, Halmemies-Beauchet-Filleau et al. (2014), investigated the use of grass silage and red clover silage in diets containing a 60:40 forage-to-concentrates ratio. Four diets were fed containing a grass:red clover ratio of 1:0, 2:1, 1:2, and 0:1. In contrast to Steinshamn (2010) they found no decreasing

fat and protein contents in milk with increasing proportions of red clover, but reported increased milk PUFA concentrations and slightly increased MY for silage mixtures compared with pure grass or red clover silage.

Generally, corn silage contains 30 to 60% grain, which leads to high starch contents compared with grass silage (160–380 vs. 0 g/kg DM) and lower fiber contents (180–250 vs. 210–290 g/kg DM; LfL, 2016). The contents of ALA are also low (3–4% of total FA) but corn silage is rich LA (50–60%) and OA (>20%), which might be the cause for a strongly increased ratio of *n-6/n-3* FA (*n-6 = omega-6 FA*) in milk (Chilliard et al., 2001b; Ferlay et al., 2006). In a recent study of van Gastelen et al. (2015), the replacement of grass silage by corn silage in diets containing an 80:20 forage-to-concentrate ratio was examined. The forage component of the four experimental diets contained increasing proportions of corn:grass silage (0:1, 1:2, 2:1, 1:0) and feed intake was restricted to 95% of ad libitum intake. Milk protein and lactose contents increased with increasing proportions of corn silage. The overall concentrations of milk SFA, MUFA, and PUFA remained constant, which is consistent with the findings of Chilliard et al. (2001b). Replacing grass silage with corn silage led to decreasing concentrations of OCFA, BCFA, CLA, and ALA and to increasing proportions of C18:1 isomers (except OA) and LA. The *n-6/n-3* ratio increased from 2.2 to 4.8 for 0 and 100% corn silage, respectively. The increasing proportions of C18:1 isomers (esp. *trans* isomers) indicate that ruminal biohydrogenation was performed less completely (van Gastelen et al., 2015). Similar results were previously obtained by Ferlay et al. (2006), who also found increased LA and CLA contents and decreased ALA contents comparing corn silage to ryegrass silage. Despite the high OA contents in corn silage, neither of the studies reported elevated milk OA, which might be a result of ruminal biohydrogenation converting OA into stearic acid (Ferlay et al., 2006; Chilliard et al., 2007; van Gastelen et al., 2015). The degree of maturity of corn silage also affects milk FA composition: feeding corn silage with increasing maturity decreases ALA concentrations and increases *n-6*/*n-3* ratio in milk fat, which might be due to decreasing ALA content and lower degradation of the leaves and the increasing proportions of starch- and LA-rich grain in mature corn silage (Khan et al., 2012).

2.3.2.3 Forage-to-Concentrate Ratio

Not only the source of forage but also the amount of concentrates in the diet affects milk FA composition. High starch diets can cause a shift to amylolytic strains of rumen microbes, reduce ruminal pH, and thus reduce biohydrogenation processes (Doreau and Ferlay, 1994). It also leads to an acidic fermentation where less acetate and more propionate is formed, which can impair milk fat synthesis in the mammary gland and thus induce milk fat depression (Ashes et al., 1997). Similar to milk FA responds to grass vs. corn silage, the concentrations of OCFA, BCFA, CLA, and ALA decrease with increasing proportions of concentrate, while LA

concentrations increase and thus the *n-6/n-3* ratio also increases. This was confirmed by Patel et al. (2013) who compared diets containing either 50, 70, or 85% grass silage on DM basis. These changes are mainly caused by low ALA and high LA concentrations in concentrate components (esp. grains). Furthermore, high grain diets induce changes in the ruminal biohydrogenation pathways to the effect that vaccenic acid as the predominant *trans* C18:1 isomer is replaced by C18:1t10 (Bauman et al., 2001; Jurjanz et al., 2004). A shift towards more SCFA, as reported by Chilliard et al. (2007) for increasing concentrate in the low range (below 60% of concentrates in total DM), was not observed by Patel et al. (2013), except for C10:0. However, another study, comparing forage-to-concentrate ratios of 60:40 and 40:60 (with or without flaxseed), found decreased C4–C8 concentrations and increased C12–C14 concentrations relating to increased amounts of concentrates (Neveu et al., 2013). Contrary to the findings of Patel et al. (2013), Neveu et al. (2013) observed an increase in CLA for the high concentrate diet, which might be due to the overall higher levels of concentrates in this study, than the other. Chilliard et al. (2007) suggested that different milk FA responses depend on the level on which the concentrate proportions are compared: increasing concentrate proportions in a rather low range (3–35%) resulted in increased *de novo* synthesized FA, LA, and *trans* 18:1 isomers, without vaccenic acid, concentrations and decreased OA, vaccenic acid, rumenic acid, and ALA in the milk of grazing cows (Bargo et al., 2002; Bargo et al., 2006). On the other hand, changes in a higher range (36–66% concentrates, with hay as forage) induced an increase of rumenic acid, LA, and *trans* C18:1 isomers and a decrease of myristic, palmitic, and stearic acids (Loor et al., 2005).

The source of starch in concentrates may also be a source of variation in milk FA by changing the rumen biohydrogenation pattern. Potatoes, containing slow degradable starch, caused an increase in rumen pH, milk fat content, and *de novo* synthesized FA and a decrease of C18:1t10, OA, LA, and ALA when replacing the same amount of wheat in the diet (30% DM), which contains rapidly degradable starch (Jurjanz et al., 2004).

2.3.2.4 Fat Supplements

As mentioned in section 2.1.4.1, dietary fat supplementation is used to increase the energy supply and can manipulate milk fat contents. Fat supplements are also subject to scientific interest concerning their potential to alter milk fat composition.

Saturated Fatty Acids

The supplementation of SFA, for example in the form of palm fat, can serve as additional source of energy without having appreciable effects on ruminal fermentation. Yet, the effects on milk performance are scarce. Furthermore, SFA supplementation is no feasible tool for improving milk quality as it favors SFA in milk fat, particularly palmitic acid in the case of palm

fat, which is undesirable for human nutrition (Palmquist et al., 1993; Chilliard et al., 2000b; Kliem and Givens, 2011). Thus, the supplementation of UFA would be the most favored means if it were not for the problems UFA cause in the rumen and for biohydrogenation, which strongly extenuates the direct transfer into milk. In general, the total concentration of unprotected fat in the diet should not exceed 4% of DM to avoid disturbance of ruminal fermentation processes (Jeroch et al., 2008). In order to achieve more consistent and stronger impacts on milk fat, a composition of rumen protected fats are used. Prerequisites for a successful rumen protection of a lipid product are "(1) consistent and predictable enhancement of unsaturated fatty acid flow to the duodenum above background, (2) adequate release and absorption of the unsaturated fatty acids in the intestines, and (3) minimal adverse effects on ruminal fermentation" (Jenkins and Bridges, 2007).

Unsaturated Fatty Acids

Popular sources of UFA are oilseeds like rapeseed, linseed, soybeans, and sunflower seeds. While soybeans and sunflower seed mainly contain LA, rapeseed and linseed are particularly rich in OA and ALA, respectively (Table 2). Oils of these seeds are the least rumen protected form, while whole seeds are considered to provide some rumen protection due to their hard seed coat and the fat being imbedded in the cell structure (Chilliard et al., 2007). Furthermore, oilseed supplements are commonly processed (extruded, roasted, cracked, etc.) which can also result in a certain level of rumen protection. Providing vegetable oils up to 4% of DM to cows does usually not lead to decreased DMI (Loor et al., 2005; Bu et al., 2007; Ye et al., 2009), can increase MY (Bu et al., 2007; Ye et al., 2009), and may cause decreased milk fat concentrations (Ye et al., 2009). If oil concentrations in the diet exceed 4%, reduced DMI und thus reduced MY accompanied by milk fat depression can occur (Chilliard et al., 2009). The application of vegetable oils also impacts milk fat composition as expected: MUFA and PUFA and *trans*-FA concentrations increase at the expense of SFA and *de novo* synthesized FA (Bu et al., 2007; Chilliard et al., 2009; Dai et al., 2011; Altenhofer et al., 2014; Hoffmann et al., 2016; Pi et al., 2016). This was recently shown by a study which aimed to improve milk fat quality for human nutrition: Pi et al. (2016) supplemented dairy cow diets with 4% rubber seed oil, 4% flaxseed oil or an even mixture of 2% flaxseed oil plus 2% rubber seed oil. They achieved strongly increased milk CLA yields by 20.9, 28.4, and 28.0 g/d for the three treatments compared with control (4.8 g/d), respectively. A similar pattern was shown for ALA and vaccenic acid, only in a lower range. The proportions of SFA were significantly decreased for the oil supplements (48.4–49.9 g/100 g FA) compared with control (70.4 g), while UFA concentrations were accordingly increased (50.1–51.6 g/100 g FA) in contrast to control (29.6 g).

In the rumen, unprotected FA in raw oils are hydrogenated very quickly and therefore partly incomplete, which explains the high amounts of intermediates of biohydrogenation in milk

(Chilliard et al., 2009; Sterk et al., 2012). FA of raw seeds are released considerably slower in the rumen which allows a more complete biohydrogenation and results in increased C18:0 concentrations and decreased biohydrogenation intermediates compared with oils or extruded seeds (Gonthier et al., 2005; Chilliard et al., 2009). However, whole linseed did not increase milk UFA concentrations to the same extent as linseed oil or extruded linseed did. Differences to the control treatment were significant but numerically rather low for SFA decrease (-2.7% points) and MUFA increase (+3.8% points) compared with extruded linseed and linseed oil (-15.2, -26.6 and +12.5, +22.3% points, respectively; Chilliard et al., 2009). The concentrations of PUFA were significantly decreased for the whole linseed treatment compared with the other supplements and even with the control (Chilliard et al., 2009). This suggests that the total amount of FA from whole linseed released and digested is rather low and a majority of the seeds might be excreted wholly. Therefore, raw oilseeds are usually ground to make FA more accessible to digestion and to achieve significant effects on milk composition (Kennelly, 1996; da Silva et al., 2007). In contrast, Oba et al. (2009) reported that whole linseed was as effective as rolled linseed in increasing milk ALA. Hoffmann et al. (2016) compared supplementation of rapeseed oil and crushed rapeseeds. They also concluded that crushed seeds may provide a certain degree of rumen protection to FA, as the milk fat of cows fed crushed rapeseeds contained more UFA than the ones fed rapeseed oil.

The use of extruded seeds shows similar or less pronounced effects than the respective oils (Ye et al., 2009), as the release of FA is slower in the rumen, but still they are far more exposed to biohydrogenation than when fed as whole seeds (Chilliard et al., 2009; Ye et al., 2009). However, the heat-treatment protects a certain amount of FA from biohydrogenation, which is proved by the highest ALA contents in milk if extruded linseed is fed compared with oil or whole linseed (Chilliard et al., 2009).

Table 2 Mean fatty acid composition (FA; g/100 g of total FA) of oilseed supplements (adapted from Glasser et al., 2008)

Item	studies	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Linseed	(n = 22)	0.1	6.1	0.1	3.4	18.8	16.3	54.4
Rapeseed	(n = 24)	0.2	4.8	0.3	2.1	60.5	20.8	9.2
Soybean	(n = 44)	0.1	11.4	0.1	4.1	22.3	53.5	7.0
Sunflower seed	(n = 13)	0.1	5.1	0.1	4.3	21.6	66.8	0.2

Protected Fats

Protected fats are tolerated in the diet by up to 6% of DM (Jeroch et al., 2008). Several techniques were developed to protect dietary fat from the rumen environment. The encapsulation of FA is one way, for example by sheathing FA or fat prills with a protein-

formaldehyde matrix or high-melting point SFA or by direct treatment of oilseeds with formaldehyde (Fievez et al., 2007; Jenkins and Bridges, 2007). Another method for rumen protection is altering the chemical structure of FA. This can be achieved by blocking the carboxyl group and thus preventing biohydrogenation by bonding with calcium or amines, forming calcium salts of FA or acyl amides, respectively. For more detailed information concerning techniques of protection and rumen biohydrogenation, the reader is referred to Jenkins and Bridges (2007). Calcium salts of FA are commercially produced sources of protected FA, which makes them an easily accessible subject of investigation. The dissociation of calcium salts requires a low pH and, therefore, should not occur until they reach the abomasum (Doreau and Ferlay, 1994). Especially calcium salts of CLA are often used for the manipulation of the milk fat content (Giesy et al., 2002; Piperova et al., 2004; Gervais et al., 2005). Petit (2003) compared the effects of raw to formaldehyde-treated linseed and sunflower seeds and found no significant differences in milk composition. Encapsulation with aldehyde treated protein seems to result in better rumen protection of fats as shown by Tymchuk et al. (1998), who, supplementing canola seeds, reported increases in milk LA and ALA concentrations by 76 and 123%, respectively. Similar results were obtained by Ashes et al. (1992) and Delbecchi et al. (2001). Lipid encapsulation or amides of CLA showed similar impacts on milk FA profile, reducing the *de novo* synthesized FA, enhancing UFA concentrations, and decreasing milk fat content (Perfield et al., 2004; Moallem et al., 2010). Jenkins (1998) studied the supplementation of high oleic canola oil or oleamide, an amide of OA and ammonia, fed at 3.5% of DM in the diet to dairy cows. Significant increases of milk OA concentrations, mainly at the expense of palmitic acid and *de novo* synthesized FA, were reported for both canola oil (35.1% of total FA) and oleamide (48.2%) compared with the control (23.2%). Milk stearic acid contents were significantly lower for oleamide than for canola oil, which indicates a markedly lower ruminal biohydrogenation. A subsequent study investigated the limits of oleamide supplementation, comparing diets which contained 0, 1, 2, 3, 4, or 5% of oleamide in DM (Jenkins, 1999). DMI was strongly decreased (-8 kg) from 0 to 5% oleamide, as were milk fat concentrations and MY. The authors concluded that the application of 2 to 3% oleamide should not be exceeded in order to prevent detrimental effects on production.

Marine Oils

Marine oils are rich in very long-chain PUFA, particularly the *n-3* FA eicosapentaenoic acid (EPA, C20:5c5,c8,c11,c14,c17, up to 32% of total FA) and the docosahexaenoic acid (DHA, C22:6c4,c7,c10,c13,c16,c19, up to 25% of total FA; Chilliard et al., 2001b). These FA belong to the group of *n-3* FA, which are regarded as desirable FA for human nutrition. Cows' milk is very poor in EPA and DHA. The supplementation of marine oils already decreases the milk fat content in low doses (50–100 g/d, mechanism not fully clear yet), and decreases

concentrations of palmitic or stearic acid and OA (Chilliard et al., 2001b). Similar results were recently achieved in fish oil supplemented dairy ewes (Carreño et al., 2016).
The transfer of EPA and DHA to milk is very low, as they are extensively (but usually not completely) hydrogenated in the rumen (Chilliard et al., 2000b) and they seem to be incorporated preferentially into plasma lipid fractions which are not available to the mammary gland (Offer et al., 2001). However, marine oils in doses of 200–300 g/d are potent to increase milk CLA contents to up to 2.7% of total FA which makes them still interesting for milk FA manipulation (Chilliard et al., 2001b). On the other hand, this increase in CLA is also accompanied by strong increases in *trans* C18:1 FA in milk (Shingfield et al., 2003). When feeding formaldehyde treated tuna oil (approx. 600g/d) to dairy cows, Kitessa et al. (2004) achieved a strong enrichment of EPA and DHA in milk fat, with 0.6 and 1.1% of total FA, respectively, while milk fat of cows to which the control treatment was fed contained none of both. Additionally, none of the aforementioned detrimental effects of supplementing untreated marine oils were observed. Protein encapsulated fish oil supplements containing either predominantly EPA or DHA did also increase milk proportions of the respective FA. They did not impair MY or milk fat concentrations but resulted in strongly increased proportions of *trans* C18:1 (Gulati et al., 2003). For the application of marine oils, benefits and detriments have to be weighed.

Several meta-analyses were conducted to summarize the effects of fat supplements on lactation performance and milk composition of dairy cows (Jenkins and Bridges, 2007; Glasser et al., 2008; Rabiee et al., 2012; Sterk et al., 2012). The effects of lipid supplements on milk fat contents and milk fat composition depend on their own FA profile, their level of saturation, the administration form, and the quantity used. Rabiee et al. (2012) ascertained that fat supplementation generally results in increased MY, milk fat concentration and yield, while DMI and milk protein concentrations decrease. Yet, they concluded that production responses were highly heterogeneous and further investigation is needed to specify the sources of variation in production responses to supplemental fat. In many cases, significant but numerically rather low effects of protected fats on milk FA profile were observed. The meta-analysis of Jenkins and Bridges (2007) assessing 25 studies showed that rumen losses and duodenal flows of PUFA were not significantly different for unprotected and chemically protected fats. Still, duodenal flows were increased for oilseed application (raw or processed), which is mainly supposed to be caused by increased intake of the respective FA. The most effective way to protect fats from ruminal biohydrogenation seems to be emulsification and encapsulation in a matrix of formaldehyde treated protein by now. But even this technology provides a protection of only about 65% (Ashes et al., 1992; Chilliard et al., 2001b). Therefore, Jenkins and Bridges (2007) concluded that there is still room for improvement of rumen protection techniques of fat supplements.

All things considered, milk fat composition is highly variable and influenced by various exogenous and endogenous factors. Especially diet composition, lipid supplements in particular, can influence milk fat concentration and composition depending on their own FA profile, their degree of saturation, presence and nature of rumen protection, and the amount supplemented. Effects of physiological state of dairy cows on milk fat composition were nearly exclusively studied under standardized conditions: animals of the same breed were housed in the same barns, fed the same diets or few diets were compared. The question remains, whether some effects of NEB on milk FA profile can be detected without detailed respect to other influential factors.

3 MATERIAL AND METHODS

3.1 General study description

The present thesis was set up as a subproject of the European Interreg VI B project “OptiMIR” (www.optimir.eu) which aimed to develop new tools for dairy farmers from spectra of mid infrared spectroscopy (MIR) obtained at milk recording.

The goal was to obtain about 400 single day milk samples from cows between the first and the twentieth week of lactation with a wide range of variance concerning their diets, origin, breeds, and season, hence having also a wide range in EB. Data of six experimental dairy farms in Germany were involved, providing altogether 379 milk samples of 313 animals and their respective data for feed intake and diet composition. The milk sampling and respective data collection took place between June 2011 and May 2012 to include possible seasonal effects on milk composition.

3.2 Experimental Stations and Animals

The experimental dairy farm “Meiereihof” of the University of Hohenheim in Stuttgart was frequented seven times, at June 8, 2011, September 12, 2011, October 8, 2011, January 10, 2012, February 7, 2012, March 14, 2012, and April 10, 2012. “Lehr- und Versuchsanstalt Hofgut Neumühle” (Neumühle) in Münchweiler was frequented three times, at July 11, 2011, October 25, 2011, and March 19, 2012. The remaining four stations “Zentrum für Tierhaltung und Technik” (Iden) in Iden, “Lehr- und Forschungsstation Frankenforst“ (Frankenforst) in Königswinter, “Versuchsgut Karkendamm“ (Karkendamm) in Bimöhlen, and “Landwirtschaftliches Zentrum Baden-Württemberg“ (Aulendorf) in Aulendorf were visited once, at August 21, 2011, September 19, 2011, February 20, 2012, and April 29, 2012, respectively. Most samplings took place during ongoing feeding trials (Table 3).

All trials, which took place during the milk samplings, did not involve any extreme treatments, which was very important for this study, as the animals were supposed to be healthy and extraordinary physiological reactions (e.g. by induced acidosis or by deficiency of a certain nutrient) were not desirable.

All sampled animals were kept in free stall barns and had ad libitum access to feed and water. They were chosen for sampling by their state of lactation, in a range of 5 to 140 DIM, irrespective of parity. The experimental stations have HF cows exclusively, except for Aulendorf, where Simmental cows are kept, and Meiereihof keeping seven Jersey cows in addition to HF cows. In total 27 Simmental cows, 6 Jersey cows and a majority of 280 HF cows

were sampled. Some animals from experimental stations which were frequented repeatedly were sampled two, three, or four times, as they matched the conditions for being sampled at more than one sampling date. However, as there were at least 27 days between two samplings, the samples were regarded as independent.

Table 3 Sampling dates, ongoing feeding trials, numbers of sampled animals, their range of lactations, and days in milk (Mean ± SD)

Station	Sampling Date	Feeding trial	Animals sampled	Parity	Days in milk
Meiereihof	June 8, 2011	no	16	1–4	77 ± 43
Neumühle	July 11, 2011	yes	30	1–6	73 ± 38
Iden	August 21, 2011	yes	45	1–8	88 ± 20
Meiereihof	September 12, 2011	yes	25	1–5	68 ± 36
Frankenforst	September 19, 2011	no	22	1–6	73 ± 42
Neumühle	October 25, 2011	yes	34	1–6	81 ± 36
Meiereihof	November 8, 2011	yes	17	1–5	76 ± 40
Meiereihof	January 10, 2012	no	20	1–5	70 ± 32
Meiereihof	February 7, 2012	no	15	1–5	78 ± 34
Karkendamm	February 20, 2012	no	69	1–6	68 ± 33
Meiereihof	March 14, 2012	no	13	1–4	86 ± 39
Neumühle	March 13, 2012	yes	33	1–6	79 ± 33
Meiereihof	April 10, 2012	no	13	1–5	55 ± 44
Aulendorf	April 29, 2012	yes	27	1–8	82 ± 25

3.3 Sampling and Parameters Recorded

3.3.1 Milk Sampling

The milk samples were collected individually for each cow under practical milk recording conditions at the evening milking of the respective sampling day and in the next morning. The sampling in Iden was exceptional because the milking was not performed twice but three times a day. As a consequence, three milk samples per cow were collected with an interval of eight hours.

Automatic sampling devices were used, which are applied at milk recording by default and set an aliquot aside into a separate vessel during the whole milking process. The milk samples from each milking were stored separately in 250 ml polyethylene bottles and kept cooled below 10°C until they were being further processed as soon as possible (usually the same day).

For processing the samples were warmed up to room temperature, then the morning and the evening milk of each single cow were mixed with respect to their proportion of the total MY while being carefully and constantly stirred. Four 50 ml polyethylene bottles were filled with

25 ml milk each and stored frozen at -18 °C prior to further analysis. Additionally, two bottles containing sodium azide as a preservative were filled with 35–40 ml of milk. In order to obtain mid-infrared spectra from Bentley as well as Foss analyzers for the OptiMIR project, one bottle each was sent by overnight express to the laboratory of the "Milchprüfring Baden-Württemberg e.V." (MPR BW) in Kirchheim/Teck and to Ravensburg (samples of 2011) or to the milk recording organization "Landeskontrollverband Nordrhein-Westfalen e.V." (LKV NRW) in Krefeld (samples of 2012).

3.3.2 Milk Component Analyses

The content of fat, protein, lactose, and urea of the preserved milk samples was analyzed by MIR; the SCC was determined by flow cytometry. For this purpose, LKV NRW used four CombiFoss analyzers, which combine either Milkoscan FT+ and Fossomatic FC or Milkoscan FT 6000 and Fossomatic 5000 analyzers (Foss, Hillerød, Denmark) for MIR spectroscopy and flow cytometry, respectively. The laboratory of MPR BW in Ravensburg also employed Foss analyzers (Milkoscan FT 6000), whereas the central laboratory in Kirchheim/Teck used Bentley FTS and Somacount FC (Bentley Instruments, Chaska, Minnesota, USA). In the course of this procedure all obtained MIR spectra were also saved and transferred to the database of LKV BW (Landesverband Baden-Württemberg für Leistungs- und Qualitätsprüfungen in der Tierzucht e.V.).

In order to help improving an existing calibration equation for the estimation of certain milk FA directly from MIR spectra (Soyeurt et al., 2011), the spectra of all samples were sent to the University of Liège, Belgium, where 143 samples were chosen for gas chromatographic milk FA analysis (GC). Another 105 samples were selected for GC to achieve a normal distribution of the EB values across the dataset.

For the analysis of the milk FA content by GC, the 248 milk samples were sent to Walloon Agricultural Research Centre (Gembloux, Belgium) via overnight express, using dry ice in order to keep the samples below the freezing point. The GC analysis was performed according to ISO 16958 (ISO, 2015) based on the method of Golay et al. (2006) using a Shimadzu gas chromatograph (Kyoto, Japan). The FA were expressed in g/100 g milk fat in the range of C4 to C22.

3.3.3 Additional Data Collected

The experimental stations provided the animals' MY and feed intake (fresh matter basis) data at of the day of sampling and the preceding seven days, except for Karkendamm, where only five days could be provided. Milk yield was recorded automatically at milking. Each experimental station uses feeding troughs with electronic animal identification and incorporated digital scales for the record of the individual feed intake. Diet composition and respective nutrient contents and animal information like DIM, parity, date of birth, and the animal ID, were also collected.

The BW was measured in different ways at the experimental stations: at Meiereihof, Karkendamm, and Frankenforst the animals automatically pass a digital livestock scale after milking. At the remaining experimental stations the animals were weighed once manually at the day of sampling or the day before.

3.4 Diets and Nutrient Composition

Table 4 shows the composition of all diets. Except for one, all experimental stations used total mixed rations (TMR). Frankenforst used a partial mixed ration with an individual, performance based addition of concentrates delivered by an automatic feeder, which explains the low content of concentrates in diet FF1.

While the animal feeding at Frankenforst and Karkendamm (KA1) was running at standard mode, a trial was taking place which aimed to replace corn silage (AU2) by pressed beet pulp silage (AU1) at Aulendorf. At Neumühle two milk samplings (October 25, 2011 and March 19, 2012) were made right before and after a trial on the application of DDGS where the respective control diet was fed exclusively (NM2). The diets ID1 and ID2 at Iden were nearly identical, except for a 1% urea supplementation in ID2, while ID3 contained additional soybean meal.

By default two different diets were applied at Meiereihof in the first third of lactation: MH1 was fed until about 35 days after calving and MH2 from that time onwards. For additional fiber supply, all animals had ad libitum access to hay as long as they were not involved in a trial. These two diets occurred in all seven samplings and were analyzed several times. The differences in the composition of single diets of the same kind were marginal, usually owing to changes of single feed stuff batches or their DM content. For this reason the diets are shown as means of their composition and nutrient contents. In September and November 2011, a feeding trial took place which aimed to compare three dried distillers' grains with solubles having diverse origins (Westreicher-Kristen et al., 2014). The diet composition of all three diets

Table 4 Composition of diets applied at the experimental stations during the milk samplings (in % DM)

Diet	MH1	MH2	MH3	MH4–6	MH7	NM1	NM2	ID1/2	ID3	FF1	KA1	AU1	AU2
Grass silage	15.7	23.4	21.3	21.3	23.7	28.5	15.9	35.3	35.2	34.6	23.1	28.7	28.8
Corn silage	21.0	19.1	15.9	15.9	17.6	28.6	15.8	37.5	37.4	42.4	34.0	.	29.6
Alfalfa silage (KA1: hay)	.	.	.	.	.	.	9.1	.	.	.	3.7	.	.
Grass Hay	15.6	13.2	15.9	15.9	17.6	4.1	1.6	.	.	.	.	.	.
Straw	1.0	2.1	.	.	.	.	3.2	1.8	2.0	2.0	.	3.3	3.3
Concentrates[1]	41.2[1]	33.7[1]	.	.	.	.	.	13.2[2]	14.5[2]	0.3[1]	29.4[1]	17.9[2]	17.9[2]
Barley	.	.	14.9	13.7	5.5	13.8	10.1	.	.	.	.	.	.
Corn grain	.	.	14.9	13.7	16.0	13.8	9.7	.	.	.	.	.	.
Extracted soybean meal	.	.	.	.	.	4.6	.	.	4.0	5.2	7.3	.	.
Extracted rapeseed meal	3.1	3.1	14.9	.	17.6	4.6	17.1	9.9	4.4	5.2	.	14.6	14.6
DDGS[3]	.	.	.	17.3	.	.	.	.	.	.	.	.	.
Brewers' yeast	.	0.5	.	.	.	.	.	.	.	.	.	.	.
Ensiled brewers' grains	.	.	.	.	.	.	.	.	.	.	.	4.4	4.4
Pressed beet pulp silage	.	.	.	.	.	.	15.6	.	.	.	.	29.7	.
Dried molasses beet pulp	.	.	.	.	.	.	.	.	.	9.5	.	.	.
Dried beet pulp	.	3.1	.	.	.	.	.	0.9	1.3	.	.	.	.
Rumen-protected fat	.	.	.	.	.	.	0.7	0.6	0.4	.	.	.	.
Additives[4]	2.3	1.8	2.4	2.4	1.9	1.9	1.3	0.8	0.8	0.8	2.5	1.3	1.3

MH = Meiereihof; NM = Neumühle; ID = Iden; FF = Frankenforst; KA = Karkendamm; AU = Aulendorf

[1] dairy concentrate based on cereal grains, protein concentrates and mineral mix; [2] energy concentrate based on cereal grains, beet pulp, and mineral mix; [3] dried distillers grains with solubles; [4] mineral feed, vitamin, feed lime, and/or salt supplement

was identical, except for the origin of the dried distiller's grains with solubles. Therefore, they are shown as only one diet in Table 4 (MH4–MH6). The control diet MH3 contained rapeseed meal for protein supply instead of dried distillers' grains with solubles. The diet MH7 was part of a feeding trial which aimed to investigate the digestibility of phytate phosphorus depending on the source of phosphorus in the diet (Haese et al., 2014).

The information about the nutrient composition of the diets (Table 5) was directly adopted from the experimental stations and therefore combined various methods. Main fractions like crude protein (CP), crude fiber (CF), and CL were analyzed by wet chemistry. Usually, the utilizable crude protein at the duodenum (uCP, g/kg DM), metabolizable energy (MJ ME) and net energy for lactation (MJ NEL) were also displayed as a result of the feed analysis either calculated from crude nutrients or from *in vitro* gas production.

Table 5 Dry matter (DM, g/kg fresh matter), nutrient (g/kg DM), and energy content (MJ/kg DM) of the diets used at the experimental stations

Diet	DM	OM	CP	uCP	CF	CL	RNB	ME	NEL
MH1	413	926	149	154	174	37	-0.8	11.4	7.0
MH2	419	915	147	152	177	38	-0.8	11.3	6.9
MH3	410	921	143	156	165	30	-2.0	11.1	6.9
MH4–6	409	926	146	156	156	37	-1.7	11.4	7.1
MH7	430	920	154	160	167	30	-0.9	11.8	7.3
NM1	491	934	148	149	158	34	-0.1	11.0	6.8
NM2	468	965	181	165	194	34	2.4	11.0	6.7
ID1/2	501	936	149	157	171	39	-1.3	11.4	7.0
ID3	506	937	150	155	168	36	-0.7	11.5	7.0
FF1	448	924	152	150	183	26	0.4	10.9	6.6
KA1	481	936	181	185	152	39	-0.6	11.4	7.1
AU1	438	922	164	165	178	25	-0.2	11.7	7.2
AU2	464	929	167	166	153	36	0.2	11.7	7.2
FF conc	894	932	194	149	166	45	7.2	12.5	7.8
MH hay	860	898	109	119	308	22	-1.7	9.1	5.3

MH = Meiereihof; NM = Neumühle; ID = Iden; FF = Frankenforst; KA = Karkendamm; AU = Aulendorf; MH hay = ad libitum hay; FF conc = individual dairy concentrate supplement; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; CF = crude fiber; CL = crude fat; RNB = ruminal N-balance; ME = metabolizable energy; NEL = net energy for lactation.

Iden, Aulendorf, and Karkendamm provided individual nutrient analyses of the feed ingredients, while Neumühle and Meiereihof had analyzed the TMR. This data had to be harmonized which included calculating some diets' nutrient composition from the wet chemical analyses of single ingredients or in a few cases estimating the nutrient composition of individual feedstuffs from tabular values (DLG, 1997). Frankenforst provided the nutrient composition of the grass and corn silage and the dairy concentrates, the remaining components were adopted

from DLG (1997). For Karkendamm the nutrient composition of the soybean meal and the alfalfa hay were also estimated from DLG (1997). In case the uCP content was missing, it was calculated using the formula of GfE (2001):

$$uCP\ (g) = \left(11.93 - \left(6.82 \times \frac{UDP\ (g)}{XP\ (g)}\right)\right) \times ME\ (MJ) + 1.03 \times UDP\ (g) \quad (1)$$

For rumen-undegraded protein (UDP) exclusively tabular values of individual feedstuffs were used (DLG, 1997).

For Diet NM2 the ME content was calculated according to Steingass and Menke (1987) and the GE (gross energy) and NEL contents were calculated by the formulae of GfE (2001):

$$ME\ (MJ) = 1.24 + 0.1457 \times GP + 0.0070 \times CP(g) + 0.0224 \times CL\ (g) \quad (2),$$

where GP = gas production in ml/200 mg DM in 24 h.

$$GE\ (MJ) = 0.0239 \times CP\ (g) + 0.398 \times CL\ (g) + 0.0201\ CF\ (g) + 0.0175\ NfE(g) \quad (3)$$

$$NEL\ (MJ) = 0.6 \times \left(1 + 0.004 \times \left(\frac{ME\ (MJ)}{GE\ (MJ)} \times 100 - 57\right)\right) \times ME\ (MJ) \quad (4)$$

3.5 Data Processing

The data directly obtained from the experimental stations, like animal information, feed intake, BW, and MY were combined in one table using MS Excel 2007 (Microsoft Corporation, Redmond, Washington, USA). By multiplying the animals' fresh matter intake with die corresponding DM content and then with the nutrient and energy contents of the diet, the DMI and nutrient/energy intakes were calculated for each animal and day. If animals had access to different diets – as it was the case at Meiereihof, where the animals had additional access to hay or, when they were adapted from diet MH1, to MH2, and at Frankenforst, where cows were supplied with individual amounts of dairy concentrates – the single nutrient intakes were summed up for each day. Following this, the intake, BW, and MY were pooled to a week's mean (of the week prior to sampling, MYw), respectively, in order to smoothen daily variations. MYw was supposed to reflect the general performance level of the cow for the calculation of ECM and then EB, while the MY at the day of milk sampling (MYs) was provided as potential predictor.

The dataset was completed by the milk recording data and the GC results for each milk sample using SAS software (SAS Institute Inc., Cary, North Carolina, version 9.3). If more than two

MY or feed intake values of the week prior to sampling or milk recording data were missing, the respective observations were removed from the dataset, which led to a dataset containing 248 observations, including results of milk FA analysis.

Furthermore, additional variables were calculated using SAS:
To show the nutrient composition per kg DM of the diet the animals had actually consumed, the average nutrient and energy intake were divided by the average DMI.

The cows' EB (MJ NEL) was calculated from the NEL intake, the NEL requirement for maintenance (MJ Mreq), and the production of ECM as proposed by GfE (2001).

$$Mreq\ (MJ) = 0.293 \times BW^{\,0.75}\ (kg) \tag{5}$$

The ECM is a "standard milk" containing 4.0 % fat and 3.4 % protein. It is used to make the milk performance of animals with different milk composition energetically comparable.

$$ECM\ (kg) = \frac{(0.38 \times fat\ (\%) + 0.21 \times\ protein\ (\%) + 0.95)}{3.18} \times MYw(kg) \tag{6}$$

As energy requirement for the production of 1 kg ECM the GfE (2001) recommends 3.28 MJ NEL, which results in a formula for the EB as follows:

$$EB\ (MJ\ NEL/d) = intake\ (MJ\ NEL/d) - Mreq\ (MJ\ NEL/d) -\ ECM\ (kg)$$
$$\times\ 3.28\ (MJ\ NEL) \tag{7}$$

To depict the cow's general energy status at the time of sampling the weekly means of the respective variables were used, except for milk fat and protein which were only available at the day of sampling.

3.5.1 Fatty Acid Groups and Indices

Groups of milk FA were calculated either by default by Walloon Agricultural Research Centre and were displayed with the GC results, or calculated manually. Their composition is displayed in Table 6.

Furthermore, some indices were calculated. The ratio of *n-6* to *n-3* FA (*n-6*/*n-3*) was calculated by Walloon Agricultural Research Centre and displayed with the GC results. As with decreasing EB the body fat mobilization is supposed to increase and thus the portion of LCFA in milk increases while SCFA and MCFA decrease, the ratio of C6–C15 to LCFA (C6–C15/LCFA) was also computed (van Knegsel et al., 2005; Gross et al., 2011a). As it may be an indicator for body fat mobilization according to Craninx et al. (2008), the ratio of C15 to C17

Table 6 Composition of milk fatty acid (FA) groups

Group		FA composition
SCFA	=	C4:0 + C6:0 + C8:0 + C10:0 +C10:1
MCFA	=	C12:0 + (C12:1c+C13:0) + C13:0*iso* + C13:0*anteiso* + C14:0 + C14:1*cis* + C14:0*iso* + C15:0 + C15:0*iso* + C15:0*anteiso* + C16:0 + C16:1*cis* + C16:1*trans* + C16:0*iso* + C17:0*iso* + C17:0*anteiso*
LCFA	=	C17:0 + C18:0*iso* + C17:1 + C18:0 + (C18:1t6-t11) + (C18:1t12-t14) + C18:1c9 + C18:1c11 + C18:1c12 + (C18:1c13+c14+t16) + C19:0 + (C18:2t,t-NMID) + (C18:2c9,t13+t8,c12) + (C18:2c9,t12+t8,c13) + C18:2t11,c15+t9,c12) + C18:2c9,c12 + C20:0 + C20:1c9 + C20:1c11 + C18:3c9,c12,c15 + C18:2c9,t11 + C22:0 + C20:3*n-6* + C20:4*n-6* + C20:5*n-3*
SFA	=	C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C13:0*iso* + C13:0*anteiso* + C14:0*iso* + C14:0 + C15:0*iso* + C15:0*anteiso* + C15:0 + C16:0*iso* + C16:0 + C17:0*iso* + C17:0*anteiso* + C17:0 + C18:0*iso* + C18:0 + C19:0 + C20:0 + C22:0
UFA	=	MUFA + PUFA
MUFA	=	C10:1 + (C12:1c + C13:0) + C14:1*cis* + C16:1*cis* + C16:1*trans* + C17:1 + (C18:1t6-t11) + (C18:1t12-t14) + C18:1c9 + C18:1c11 + C18:1c12 + (C18:1c13+c14+t16) + C20:1c9 + C20:1c11
PUFA	=	(C18:2t,t-NMID) + (C18:2c9,t13+t8,c12) + (C18:2c9,t12+t8,c13) + (C18:2t11,c15+t9,c12) + C18:2c9,c12 + C18:3c9,c12,c15 + C18:2c9,t11 + C20:3*n-6* + C20:4*n-6* + C20:5*n-3*
OCFA	=	C13:0*iso* + C13:0*anteiso* + C15:0 + C15:0*iso* + C15:0*anteiso* + C17:0 + C17:1 + C17:0*iso* + C17:1*anteiso* + C19:0
BCFA	=	C13:0*iso* + C13:0*anteiso* + C14:0*iso* + C15:0*iso* + C15:0*anteiso* + C16:0*iso* + C17:0*iso* + C17:0*anteiso* + C18:0*iso*
∑C4-C15	=	C4:0 + C6:0 + C8:0 + C10:0 + C10:1 + C12:0 + (C12:1c + C13:0) + C13:0*iso* + C13:0*anteiso* + C14:0 + C14:1*cis* + C14:0*iso* + C15:0 + C15:0*iso* + C15:0*anteiso*
∑C6-C15	=	C6:0 + C8:0 + C10:0 + C10:1 + C12:0 + (C12:1c+C13:0) + C13:0*iso* + C13:0*anteiso* + C14:0 + C14:1*cis* + C14:0*iso* + C15:0 + C15:0*iso* + C15:0*anteiso*
∑C16	=	C16:0 + C16:1*cis* + C16:1*trans* + C16:0*iso*
∑C18:1*cis*	=	(C18:1t6-t11) + (C18:1t12-t14) + C18:1c9 + C18:1c11 + C18:1c12
∑C18:1*trans*	=	(C18:1t6-t11) + (C18:1t12-t14)
∑C18:2	=	(C18:2t,t-NMID) + (C18:2c9,t13+t8,c12) + (C18:2c9,t12+t8,c13) + (C18:2t11,c15+t9,c12) + C18:2c9,c12
∑*trans*FA	=	(C18:1t6-t11) + (C18:1t12-t14) + (C18:2t,t-NMID) + (C18:2c9,t13+t8,c12) + (C18:2c9,t12+t8,c13) + (C18:2t11,c15+t9,c12)
∑*n-3*	=	C18:3c9,c12,c15 + C20:4*n-6* + C20:5*n-3*
∑*n-6*	=	C18:2t,t-NMID + (C18:2c9,t13+t8,c12) + (C18:2c9,t12+t8,c13) + (C18:2t11,c15+t9,c12) + C18:2c9,c12 + C20:3*n-6* + C20:4*n-6*
n-6/n-3	=	∑*n-6* / ∑*n-3*
C6-C15/LCFA	=	∑C6-C15 / LCFA
C15:C17	=	(C15:0 + C150*iso*+C15:0*anteiso*) / (17:0 + C17:1 + C17:0*iso* + C17:0*anteiso*)
OA/*de novo*	=	C18:1c9 / (C6:0 + C8:0 + C10:0 + C12:0 + C14:0 + C16:0)

SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SCFA = short-chain FA (<C12); MCFA = medium-chain FA (C12-C16); LCFA = long-chain FA (>C16); OCFA = odd-chain FA; BCFA = branched-chain FA, t = *trans*; c = *cis; n-3 = omega-3* FA; *n-6 = omega-6* FA;

FA (C15/C17) was also calculated. Lastly, the ratio of OA to saturated straight-chain *de novo* synthesized FA (C6:0 + C8:0 + C10:0 + C12:0 + C14:0 + C16:0) was calculated for the pre-specified model (OA/*de novo*) similar to the ratio proposed by van Knegsel et al. (2005) and Gross et al. (2011a).

3.6 Statistical Evaluation

Descriptive summary statistics including the mean, standard deviation (SD), minimum, and maximum values were calculated using the MEANS procedure of SAS. Furthermore, the Pearson correlation (r) between all pairs of the potential predictor variables was computed using the CORR procedure in SAS. The percent coefficient of variation (%CV) was calculated to make variation of variables with great differences in absolute value comparable:

$$\%CV = \frac{SD}{mean} \times 100 \quad (8)$$

For the EB model development, the response variable was EB whereas the predictor variables only included DIM, parity, nutrient and NEL content of the individual diets, milk protein, fat, and lactose concentrations obtained from Bentley spectra, and the individual milk FA. Parity was coded as a two-level qualitative factor, with "1" denoting primiparous and "2" multiparous cows. The considerations which led to the inclusion or exclusion of certain predictors are discussed in section 4.2. In total 62 of the initially 86 potential predictors (see Table 12 in section 4.3) were chosen to build two variable pools: Pool1 contained all the 62 variables which were also provided to the regularized regression methods, Pool2 only contained the individual milk FA. Pool2 was created to enable the evaluation of the influence of milk FA on the accuracy of EB prediction.

3.6.1 Stepwise Variable Selection

Stepwise variable selection was used to predict EB from the two variable pools Pool1 and Pool2 in the GLMSELECT procedure of SAS.

The stepwise variable selection involved choosing the variable to be added to the model according to the following specific prescriptions: If a predictor failed to satisfy the set conditions at any stage of the variable selection process, it was removed from the current model. This procedure was executed until all variables fulfilling the defined conditions were added to the model. The criterion for entry into or retention in the model was set to a p-value <0.15. In the course of the stepwise variable selection, numerous models were generated. In order to identify the most suitable one, a five-fold cross-validation was performed.

3.6.2 Regularized Linear Regression Models

Several regularized or penalized multiple linear regression methods were used to predict EB from the full set of covariates using the *glmnet* package in "R" software (Friedman et al., 2010). The specific methods used consisted of ridge regression (ridge), the least absolute shrinkage and selection operator (Lasso), adaptive Lasso (AdaLasso), elastic net (ENET) and adaptive elastic net (ADAENET) following the approach detailed in Ogutu et al. (2012).

All the five methods use the same basic multiple linear regression model (9) to predict EB and only differ in the way they penalize β, the p-dimensional vector of regression coefficients of fixed effects:

$$y = \mu 1_n + X\beta + e \quad (9),$$

where y is the vector of observed values of EB, μ is the general mean, X is a $n \times p$ design matrix and e are normally and identically distributed normal residual errors.

The five regularization models involve minimization of the expressions given in (10) to (14) below, which also show the specific penalty applied by each method.

$$Ridge: \hat{\beta} = \underset{\beta}{\arg min} \, \|y - X\beta\|_2^2 + \lambda \|\beta\|_2^2 \quad (10)$$

$$Lasso: \hat{\beta} = \underset{\beta}{\arg min} \, \|y - X\beta\|_2^2 + \lambda \|\beta\|_1 \quad (11)$$

$$AdaLasso: \hat{\beta} = \underset{\beta}{\arg min} \, \|y - X\beta\|_2^2 + \lambda \sum_{j=1}^{p} \widehat{\omega}_j |\beta_j| \quad (12)$$

$$ENET: \hat{\beta} = \left(1 + \frac{\lambda_2}{n}\right) \left\{ \underset{\beta}{\arg min} \, \|y - X\beta\|_2^2 + \lambda_2 \|\beta\|_2^2 + \lambda_1 \|\beta\|_1 \right\} \quad (13)$$

$$ADAENET: \hat{\beta} = \left(1 + \frac{\lambda_2}{n}\right) \left\{ \underset{\beta}{\arg min} \, \|y - X\beta\|_2^2 + \lambda_2 \|\beta\|_2^2 + \lambda_1^* \sum_{j=1}^{p} \widehat{\omega}_j |\beta_j| \right\} \quad (14)$$

For methods (10) to (14) $\|y - X\beta\|_2^2$ is the residual sum of squares $=\sum_1^n (y - X\beta)^2$, $\|.\|_2$ is the L_2-norm, λ is the penalty or shrinkage parameter, $\|.\|_1$ is the L_1-norm, and $\widehat{\omega}_j$ are variable-specific shrinkage parameters.

As evident in (10) to (14), the five methods differ with respect to the regularization method used. Ridge regression (10) uses a L_2-norm penalty to shrink the regression coefficients of all the predictor variables equally towards zero and each other (Hoerl and Kennard, 1970). It is therefore suited for prediction with many covariates each of which only makes a small contribution to the prediction of the response variable. The ridge penalty does not perform automatic variable selection, leading to less interpretable, high-dimensional models (Ogutu et

al., 2012). The L_1-norm Lasso penalty (11) is able to shrink the regression coefficients similar to the ridge regression but, unlike ridge regression, it is also able to automatically select the most important and relevant covariates by shrinking some coefficients to exactly zero (Tibshirani, 1996). Despite its ability to perform automatic variable selection, the Lasso suffers from the disadvantage that it cannot select more covariates than the sample size and cannot efficiently select between two or more highly correlated covariates. The AdaLasso (12) attempts to surmount some of the shortcomings of the Lasso by allowing for a predictor-specific penalty denoted by $\widehat{\omega}_j$ (Zou, 2006). The ENET (13) tries to alleviate the weaknesses of the Lasso by combining the Lasso and the ridge penalties into a composite penalty. The ridge penalty ensures stability and low variance of the estimated regression coefficients, whereas the Lasso penalty ensures automatic variable selection (Zou and Hastie, 2005). The ADAENET (14) is constructed in the same spirit as the AdaLasso and enables variable-specific penalization and automatic selection (Zou and Zhang, 2009).

All variables were standardized to zero mean and unit variance prior to model fitting. The optimal penalty parameter for each of the five methods was selected by five-fold cross-validation. Five-fold cross-validation was also used to evaluate the predictive accuracy of the five methods. The dataset was split into five random subsets of approximately equal sizes, containing 49 or 50 observations. Four of the five subsets were concatenated and used as a training set and each remaining subset used as a validation set, in turn, for a total of five cross-validation replicates. The observed EB values were first deleted from each validation set and then predicted using each of the five regularized linear regression models trained on the corresponding training set.

The predictive accuracy was quantified using r between the predicted and the observed values and the root mean square error (RMSE) computed as (15):

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}} \tag{15}$$

Both metrics were calculated for each of the five replicate validation sets and averaged over all replicates, separately for each method.

3.6.3 Random Forests

In addition to the regularization methods described above, a random forests regression (Breiman, 2001) was used as a further machine learning method, to relate EB to the 62 predictor variables using the *randomForest* package in “R” software.

Random forests regression involves generating an ensemble of regression trees by taking a random bootstrap sample of size 248 with replacement from the data and a random subsample of 62/3 of the 62 predictors without replacement. The selected sample is used to construct a Classification and Regression Tree (CART) partition of the data. Because it uses CART random forests are useful as exploratory tool for identifying potentially nonlinear associations. At each split, a random subsample of the predictors is selected and used to determine the variable and value of the variable leading to the optimal split (Liaw and Wiener, 2002). An optimal split is one that results in minimal node impurity or mean squared error. Using a random subsample of predictors at each possible split ensures the fitted values are more independent across trees. This process is continued until the tree is as large as desired. The trees are not pruned because large trees reduce bias. The observations that are not included in the selected bootstrap sample, called the Out-of-Bag (OOB) data, are then dropped down the tree. The class assigned to each observation and each observation's predictor values are stored. The preceding steps were repeated 3000 times. More trees are better than a few. Random forests do not overfit as more trees are grown (Breiman, 2001).

The predicted value for each case is calculated as the average of the OOB observations that ends up in the terminal node containing the case. Because the OOB data is used for prediction, there is no need for test data (Breiman, 2001). The node size indicating the minimum number of observations in the terminal node beyond which the node could not be split further, was set to 5. The predicted values for each case are averaged across all trees. Since the trees are more independent because of selection of the subsets of predictors at each split, the averaging reduces variance more dramatically than if all predictors were considered at each split. All variables, including weak predictors, highly correlated predictors, or highly specialized predictors have a chance to contribute to the fit because of sampling at each split.

The importance of each predictor is quantified using randomization as follows (Liaw and Wiener, 2002). First, the prediction error for each tree is estimated by dropping the OOB data down the tree. Second, for p predictors, the first step is repeated p times but each time using the randomly shuffled (permuted) values of each predictor. Randomly shuffling (permutation) values of each predictor in the manner described makes the predictor less related to the response variable and other predictors. Third, for each of the p predictors, the difference between the prediction error with no shuffling and the prediction error with the predictor shuffled is averaged over trees to get the importance of that predictor for forecasting accuracy. The importance of the *s*-th predictor is calculated as:

$$I_s = \sum_{r=1}^{r=T} \left(\frac{1}{T} (\vartheta_s - \vartheta) \right), s = 1, 2, \dots, p \qquad (16),$$

where T is the number of trees, ϑ_s is the prediction error with predictor s shuffled, and ϑ is the prediction error with none of the predictors shuffled. The prediction error is computed as:

$$MSE_{OOB} = \frac{\sum_{i=1}^{i=n}\left(y_i - \hat{y}_i^{OOB}\right)^2}{n} \quad (17),$$

where $\hat{y}_i^{OOB}$ is the mean of the predictions of the *i*-th observation.

The variable importance therefore measures the increase in mean squared prediction error (MSE_{OOB}) or node impurity due to shuffling the values of a given predictor. The greater the increase in the prediction error due to shuffling a particular predictor, the more important is the contribution of the predictor to the forecasting skill. It is important to note that variable importance can depend on complex interactions with other variables (Liaw and Wiener, 2002). The variable importance plot produced by random forests was used to identify the subset of potentially most important predictors for consideration in further confirmatory analysis. To make the predictive performance of random forests comparable with the other methods, the Pearson correlation between the predictions based on the OOB data ($\hat{y}_i^{OOB}$) and the observed values as well as the RMSE were calculated.

3.6.4 Selection of the Best Overall Model Based on the Regularization Methods and Random Forests

The variables selected by Lasso, AdaLasso, ENET, ADANET, and the 30 top ranked variables in the random forests importance plot were further evaluated in an effort to identify a set of the most important covariates across all the models and use them to build one final linear regression model for predicting EB using the entire dataset in the procedure MIXED of SAS. For model selection the corrected Akaike Information Criterion (AICC) was used (Hurvich and Tsai, 1993):

$$AICC = -2logL + \frac{n \times (n+d)}{n-d-2} \quad (18),$$

where *L* is the Likelihood, *n* (= 248) is the number of observations or sample size and *d* is the number of the estimated parameters in the model. This criterion penalizes complexity and enforces a tradeoff between model complexity and parsimony and prefers simpler to more complex models. The relative change, not the absolute value of AICC, is used in comparing contending models. The model with a smaller AICC is preferred.

The evaluation was carried out using a five-step process. In the first step, a multiple linear regression model containing all the predictor variables selected by random forests and each

of the four regularization methods across all the five replicate validation sets (MODEL1) was fit separately for each method and AICC was computed using the maximum likelihood (ML) in MIXED.

In the second step, each individual variable in the full model in step one was used to predict EB and then the variables were sorted in ascending order by their AICC, computed by ML. The AICC-best supported variable was kept in the model and the next AICC-best supported variable was added to the model. If the resultant model had a smaller AICC then the new variable was also retained. But if the AICC increased or remained the same, then the variable was removed and the next best one added to the model. This procedure was repeated until all the variables had been tested, yielding MODEL2.

In the third step, the selected model (MODEL2) was further reduced by removing all the variables with non-significant effects ($p>0.05$), (MODEL3). In one case (AdaLasso) the second step was retrospectively skipped, as it led to no improvement, and non-significant predictors were removed directly from MODEL1, which resulted in MODEL4.

In the fourth step, all MODEL1, MODEL2, and MODEL3/4 were refit using restricted maximum likelihood (REML) and the regression coefficients estimated. This was necessary because REML estimates are more efficient than ML estimates when the number of estimated parameters relative to sample size is high (Patterson and Thompson, 1971).

Lastly, the RMSE (15), r, and the adjusted coefficient of determination (R^2_{adj}) were calculated to assess the predictive accuracy of the three preceding models.

$$R^2_{adj} = 1 - (1 - R^2) \times \frac{n-1}{n-p-1} \quad (19),$$

where n is the sample size and p is the number of estimated parameters.

Multiple metrics to assess predictive performances of models are useful because different metrics have different merits and demerits. For example, the coefficient of determination (R^2) assumes values between 0 and 1 and gives information about the proportion of the variance of a dependent variable *y* explained by a statistical model. In linear regression models it is equal to the squared Pearson correlation coefficient. The R^2 is the higher the more complex the models are, irrespective of whether or not the additional effects provide additional information. However, this can lead to overfitting, and hence it is not safe to use R^2 as the sole measure of predictive performance. The R^2_{adj} alleviates this problem by accounting for the number of estimated fixed effects in the model, just as AICC does, but unlike AICC, R^2_{adj} decreases with increasing number of estimated fixed effects.

To be able to compare the models built with GLMSELECT (section 3.6.1) with the models developed in this section, the GLMSELECT derived models were also refitted using the MIXED procedure. The AICC was then computed using ML method and the regression coefficients using REML. The RMSE, r, and R^2_{adj} were also computed for each model using the same formulae (15, 18) as described in section 3.6.2 and above, as the GLMSELECT computes the RMSE and AICC in a different way than (15) and (19).

In order to make the results of the present study comparable to others and to visualize the differences between R^2 and R^2_{adj}, the R^2 is additionally displayed with the results.

3.6.5 Leave-one-out Cross-Validation

The model with the greatest strength of support (lowest AICC) in the data between MODEL1, MODEL2 and MODEL3/4 was chosen from each selection method described in section 3.6.4 and from the models generated with GLMSELECT. In order to quantify their predictive performances, a leave-one-out cross-validation was performed using MIXED. The predicted values for EB were then again used to compute RMSE, r, R^2 and R^2_{adj} for cross-validation.

4 RESULTS AND DISCUSSION

In this section, several descriptive summaries of the studied attributes are presented. This is followed by the correlations between each of the candidate variables and the response variable (EB). The latter correlations are used to examine the strength and nature of the relationships between the response and each of the candidate variables. Subsequently, results from the different steps of model development and evaluation are presented and discussed. Finally, these results are compared briefly with those of other similar studies.

4.1 Descriptive Statistics

4.1.1 Diet Composition

Several different diets were provided to the study animals, with some individuals receiving a partial mixed ration and concentrates or having ad libitum access to extra hay. Table 7 shows the average diet composition which the animals actually consumed during the week prior to milk sampling. The diets met the requirements of early lactating dairy cows according to GfE (2001), and did not show extreme variations. On average the diets contained 451 g DM per kg FM and 6.9 MJ NEL, 162 g CP, 173 g CF, and 35 g CL per kg DM, respectively. The percent coefficient of variation (%CV) was lowest for organic matter (OM, 0.6%) and energy content (2.1–2.8%) and highest for CF (9.3%), CP (9.5%), and CL (10.8%).

Table 7 Descriptive statistics for diet composition across all observations

	MEAN	SD	%CV	MIN	MAX
DM (g/kg FM)	451.4	30.6	6.8	386.0	525.1
ME (MJ/kg DM)	11.3	0.2	2.1	10.9	11.8
NEL (MJ/kg DM)	6.9	0.2	2.8	6.6	7.3
OM (g/kg DM)	928.3	5.7	0.6	910.6	936.8
CP (g/kg DM)	161.6	15.4	9.5	142.1	183.0
uCP (g/kg DM)	161.5	11.3	7.0	149.0	184.6
RNB (g/kg DM)	0.0	1.5	-	-2.1	2.7
CF (g/kg DM)	172.9	16.0	9.3	151.4	202.0
CL (g/kg DM)	34.9	3.8	10.8	24.9	42.8

SD = standard deviation; %CV = percent coefficient of variation; MIN = minimum value; MAX = maximum value; DM = dry matter; FM = fresh matter; ME = metabolizable energy; NEL = net energy for lactation; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; RNB = ruminal N-balance; CF = crude fiber; CL = crude fat

4.1.2 Animal Performance

On average, the study animals were in their 2.6th lactation and 74 DIM, ranging from 6 to 133 days (Table 8). Their MYs (37.5 kg/d) and MYw (37.3 kg/d) were nearly identical, indicating that MYs is a suitable measure for displaying the general level of milk performance in healthy cows, and thus is also suitable as a candidate predictor variable, because the variation in MY between successive days is generally low (Syrstad, 1977; Svennersten-Sjaunja et al., 1997). ECM yield was approximately 2 kg lower than MYw or MYs, reflecting lower actual milk fat and protein contents than those used for calculating ECM (Table 9). The calculated EB showed a wide range of variation, as expected, ranging from -83.5 to 66.8 MJ NEL/d, with a mean of -6.5 MJ NEL/d.

Table 8 Descriptive statistics for animal performance (n = 248 samples)

	MEAN	SD	%CV	MIN	MAX
Parity	2.6	1.5	59.3	1.0	8.0
DIM (d)	74.0	34.1	46.0	6.0	133.0
BW (kg)	642.5	85.9	13.4	365.8	852.0
MYs (kg/d)	37.5	8.0	21.5	14.1	57.9
MYw (kg/d)	37.3	8.1	21.6	13.8	57.0
ECM (kg/d)	35.4	7.4	20.8	15.1	54.8
DMI (kg/d)	21.3	3.8	17.7	11.6	33.0
Mreq (MJ NEL/d)	37.3	3.8	10.1	24.5	46.2
Preq (MJ NEL/d)	116.2	24.2	20.8	49.5	179.7
EB (MJ NEL/d)	-6.5	26.6	-	-83.5	66.8

SD = standard deviation; %CV = percent coefficient of variation; MIN = minimum value; MAX = maximum value; DIM = days in milk; BW = body weight; MYs = milk yield at day of sampling; MYw = milk yield, week's mean prior to sampling; ECM = energy-corrected milk yield (4.0% fat, 3.4% protein); DMI = dry matter intake; Mreq = requirement for maintenance; Preq = requirement for milk performance; EB = energy balance

4.1.3 Milk Composition and Milk Fatty Acid Profile

The average milk composition across all the observations (Table 9) was 3.2% protein, 3.8% fat, and 4.9% lactose. The fat and protein concentrations were thus below the 4% for fat and 3.4% for protein, which were presumed for ECM calculation. This explains the lower ECM yield compared with MYw. According to Walker et al. (2004), the fat and protein concentrations in milk decline directly after calving, reaching their nadir at 40 to 60 DIM, when MY peaks, then increase until the end of lactation. The decrease is a consequence of increased lactose production. Milk lactose concentration is the most constant milk component because increased lactose production causes an increase in milk volume via the osmotic principle and vice versa. Thus, decreasing milk fat and protein concentrations in early lactation are caused by dilution due to enhanced milk volume. Expectedly, the %CV was lowest for lactose (3.5%), followed

by protein (8.7%) and fat (17.1%). The mean SCC was 161 k/ml, which is an acceptable value according to Barkema et al. (1998) who categorized bulk milk SCC into low (≤150 k/ml), medium (160–250 k/ml) and high SCC (250–400 k/ml). However, the values of SD and %CV were very high (516 k/ml, and 319%, respectively). Animals were only sampled if they showed no apparent symptoms of mastitis. The majority (n = 185) of the study animals had very low SCC (<100 k/ml), but 23 animals exceeded the threshold of 250 k/ml for pathological SCC recommended by Dohoo and Meek (1982). Four of these animals showed extremely high SCC values of 1228 to 5728 k/ml, which strongly contributed to the high values of SD and %CV.

Table 9 Descriptive statistics for milk composition (n = 248 samples)

	MEAN	SD	%CV	MIN	MAX
Protein (%)	3.20	0.28	8.73	2.62	4.32
Fat (%)	3.75	0.64	17.07	1.63	6.28
Lactose (%)	4.88	0.17	3.46	3.90	5.40
Urea (mg/dl)	22.35	6.06	27.10	8.00	40.00
SCC (k/ml)	162	516	319	33	5728
FPR	1.18	0.20	17.30	0.52	1.89

SD = standard deviation; %CV = percent coefficient of variation; MIN = minimum value; MAX = maximum value; SCC = somatic cell count; FPR = milk fat-to-protein ratio

The milk fat-to-protein ratio (FPR) is considered a sufficient and practical tool for assessment of energy status (Grieve et al., 1986; Buttchereit et al., 2010). Generally, a value between 1.0 and 1.5 is considered normal (Dürr and Kraft, 2005). A lower FPR is associated with the risk of acidosis to occur, because an excess supply of concentrates and a concurrent lack of fibrous constituents, which are causative for acidosis, lead to decreased ruminal acetate production, which hampers mammary *de novo* synthesis of FA and leads to milk fat depression (Ashes et al., 1997). Under these conditions milk protein levels usually stay within the normal range or slightly increase. A FPR above 1.5, however, indicates the risk of ketosis to develop. This can affect both milk fat and protein: enhanced body fat mobilization increases milk fat content and an energy deficit leads to reduced ruminal microbial protein synthesis, low uCP supply and thus to a lower milk protein content (Kirchgessner et al., 1986; Grieve et al., 1986). The mean FPR fell within the normal range (1.18) and ranged from 0.52 to 1.89.

As shown in section 2.2, there are few milk FA with concentrations higher than 10 g/100 g of total FA in bovine milk; and the vast majority have concentrations of even lower than 1 g/100 g FA. In the present dataset (Table 10), the most abundant FA were palmitic acid (C16:0; 29.1 g/100 g FA) and OA (C18:1c9; 20.1 g/100 g FA), followed by myristic acid (C14:0; 11.3 g/100 g FA) and stearic acid (18:0; 10.6 g/100 g FA).

Table 10 Descriptive statistics for individual milk fatty acids (FA; n = 248 samples)

FA (g/100 g FA)	MEAN	SD	%CV	MIN	MAX
C4:0	2.66	0.27	10.22	1.55	3.74
C6:0	1.68	0.21	12.64	1.03	2.23
C8:0	1.16	0.19	16.33	0.64	1.56
C10:0	2.83	0.62	21.84	1.22	4.30
C10:1	0.33	0.10	31.53	0.10	0.67
C12:0	3.47	0.80	23.02	1.31	5.55
C12:1*cis*+C13:0	0.22	0.07	33.13	0.06	0.50
C13:0*iso*	0.03	0.01	38.40	0.01	0.06
C13:0*anteiso*	0.08	0.03	36.96	0.01	0.17
C14:0	11.29	1.71	15.12	5.92	14.57
C14:1*cis*	0.91	0.27	30.04	0.32	1.87
C14:0*iso*	0.08	0.02	30.40	0.03	0.19
C15:0	1.24	0.34	27.55	0.51	2.98
C15:0*iso*	0.20	0.04	18.02	0.09	0.39
C15:0*anteiso*	0.44	0.09	20.90	0.18	0.73
C16:0	29.12	3.52	12.07	20.37	39.94
C16:1*trans*	0.04	0.02	37.91	0.00	0.11
C16:1*cis*	1.58	0.45	28.36	0.79	3.84
C16:0*iso*	0.22	0.06	26.38	0.11	0.67
C17:0	0.64	0.11	17.36	0.39	1.17
C17:1	0.01	0.005	62.74	0.00	0.03
C17:0*iso*	0.37	0.05	13.32	0.27	0.63
C17:0*anteiso*	0.59	0.19	31.92	0.15	0.93
C18:0	10.62	2.24	21.07	4.55	20.26
C18:0*iso*	0.07	0.02	30.45	0.03	0.14
C18:1t6-t11	1.93	0.68	35.21	0.64	7.66
C18:1t12-t14	1.13	0.32	28.05	0.53	2.36
C18:1c9	20.89	4.13	19.75	12.90	33.42
C18:1c11	1.03	0.28	26.78	0.36	1.91
C18:1c12	0.33	0.07	22.36	0.19	0.58
C18:1c13+c14+t16	0.48	0.10	21.17	0.25	0.74
C18:2t,t-NMID	0.05	0.02	41.88	0.02	0.19
C18:2c9,t11	0.37	0.11	30.61	0.18	0.84
C18:2c9,t13+t8,c12	0.23	0.07	29.35	0.09	0.47
C18:2c9,t12+t8,c13	0.26	0.10	37.85	0.12	0.58
C18:2t11,c15+t9,c12	0.24	0.08	33.20	0.00	0.61
C18:2c9,c12	1.91	0.35	18.43	1.19	3.32
C18:3c9,c12,c15	0.45	0.10	22.47	0.27	0.97
C19:0	0.11	0.05	48.90	0.02	0.36
C20:0	0.16	0.04	28.23	0.00	0.31
C20:1c9	0.13	0.02	19.04	0.08	0.24
C20:1c11	0.07	0.03	40.76	0.03	0.30
C20:3*n-6*	0.09	0.02	26.76	0.03	0.17
C20:4*n-6*	0.12	0.03	21.61	0.06	0.20
C20:5*n-3*	0.05	0.03	53.12	0.00	0.22
C22:0	0.05	0.02	46.96	0.00	0.12
C22:5*cis*	0.05	0.02	37.39	0.00	0.12

SD = standard deviation; %CV = percent coefficient of variation; MIN = minimum value; MAX = maximum value; c = *cis*; t = *trans*; t, t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA

The next group of FA with concentrations greater than 1 g/100 g FA mainly consisted of straight-chain SFA, specifically C4:0 to C12:0, and C15:0, C16:1*cis*, C18:1t6-11, C18:1t12-14 and C18:2c9,c12. All the remaining FA had concentrations lower than 1 g/100 g FA, notably 27 of the total of the 47 analyzed FA had concentrations below 0.1 g/100 g FA. As most of the milk samples were chosen for GC analysis according to their infrared spectra in order to improve the accuracy of the FA prediction model, there was apprehension that this procedure could potentially bias the dataset by choosing rather uncommon spectra und thus FA profiles. However, this seemed not to be the case. Instead, the mean FA profile and its SD were generally comparable with that for the standard milk FA profile presented in Table 1 in section 2.2. Fatty acids with very low concentrations also tended to have very high %CV of 40–62. The concentrations of C4:0 (%CV = 10.2), C16:0 (%CV = 12.1), and C6:0 (%CV = 12.6) showed the least overall variation.

The groups of FA displayed in Table 11 mostly have relatively low %CV, showing that the grouped FA are less variable than the individual ones, as the FA often compensate for each other within the grouping. This agrees with Gross et al. (2011a) who concluded that the predictive value of single FA having low concentrations is limited concerning NEB detection because of their concurrent high degree of variation. They found that changes in groups of FA were more suitable as indicators for EB because of their higher concentrations and lower variation. This might hold true for selected groups of FA but should not rule out investigating the effects of EB on single FA, if the prediction is desired to be as accurate as possible (see section 4.3 for further details).

4.2 Screening Variables for Potential Predictors

In the process of developing models for EB prediction, some variables turned out to be either unnecessary, inappropriate, or undesirable as predictor variables for various reasons: Although both Simmental and Jersey cows were sampled, breed was not considered as a predictive factor as the eight Jersey samples were too few relative to the 217 samples from HF cows to show a clearly discernible effect. Additionally, all the Simmental cows originated from the same experimental station and were fed the same diets. The possible breed effects were thus confounded with diet or station effects and hence are even harder to separate out and estimate reliably. Breed would have been more appropriate as a predictor if several other experimental stations had also provided milk samples from Simmental and/or Jersey cows.

Table 11 Descriptive statistics for grouped milk fatty acid (FA) profile and indices (n = 248 samples)

Group (g/100 g FA) or ratio	MEAN	SD	%CV	MIN	MAX
SCFA	8.67	1.06	12.24	5.17	11.18
MCFA	48.91	5.69	11.64	33.93	64.39
LCFA	42.42	6.22	14.66	26.35	60.05
SFA	67.10	4.86	7.25	51.47	77.04
UFA	32.90	4.86	14.78	22.96	48.53
MUFA	29.09	4.67	16.05	20.05	42.35
PUFA	3.81	0.60	15.81	2.72	6.18
OCFA	3.70	0.49	13.14	2.39	5.76
BCFA	2.07	0.27	13.07	1.42	3.05
∑C4-C15	26.62	3.72	13.98	14.83	34.18
∑C6-C15	23.95	3.80	15.85	12.03	31.76
∑C16	30.96	3.58	11.56	22.34	42.69
∑C18:1*cis*	22.74	4.42	19.43	14.19	36.05
∑C18:1*trans*	3.06	0.92	30.11	1.58	9.40
∑C18:2	3.06	0.52	16.90	2.10	5.30
∑*trans*	3.85	1.06	27.48	2.04	10.89
∑*n-3*	0.55	0.11	20.21	0.34	1.09
∑*n-6*	2.90	0.46	16.01	1.96	4.67
n-6/n-3	5.40	0.90	16.74	2.70	8.87
C6-C15/LCFA	0.59	0.16	27.62	0.20	1.08
C15/C17	1.18	0.27	22.88	0.51	2.03

SD = standard deviation; %CV = percent coefficient of variation; MIN = minimum value; MAX = maximum value; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA; SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SCFA = short-chain FA (<C12); MCFA = medium-chain FA (C12-C16); LCFA = long-chain FA (>C16); OCFA = odd-chain FA; BCFA = branched-chain FA

Variables used to calculate EB were excluded from consideration as potential predictors, except for MY, milk protein and fat concentration, and dietary NEL concentration because these latter variables provide easily accessible information under practical conditions and thus are valuable as potential predictors. Of the three MY measures, MYs, MYw and ECM, only MYs was retained as it is the measure most likely to be used in practical applications of a predictive model and is nearly identical to MYw, as previously stated, but not the same. Milk composition variables were available from both, Foss and Bentley analyzers. For modeling, Bentley measurements were used exclusively as Bentley provided milk lactose content for all samples, whereas the Foss data lacked lactose data with the samples analyzed in Ravensburg. Milk urea was disqualified from consideration as a predictor of EB as proposed by Kirchgessner et al. (1986) because the accuracy of its MIR prediction may be questionable due to its extremely low concentration in milk (Hanuš et al., 2008). Although generally considered a sufficient tool, FPR was not used as predictor variable, except for the pre-specified model (see section 4.4.4). With respect to single observations, a total of 137 animals were in NEB, 82 of these were lower than -15 MJ NEL/d. However, only 19 animals showed a

FPR above 1.5. In early lactation, the problems might compensate each other regarding their effects on FPR: On the one hand, the amount of concentrates is usually high, which would lead to decreased ruminal acetate production followed by decreased milk fat concentration and thus a low FPR. On the other hand, animals are still in NEB because of their high MY, which would result in a high FPR due to increased body fat mobilization (increases milk fat) and decreased milk protein production. Hence, a high FPR is more likely to indicate acute ketosis than NEB in early lactation and thus might not serve as an appropriate indicator.

Only individual FA were added to the main pool of candidate predictors because using grouped FA could not serve the purpose of identifying FA which are potentially important for the prediction of EB. Furthermore, most groups of FA showed high correlations with each other and OA, indicating that they basically provide the same information, and in some groups FA with opposite relationships to EB were summed up (e.g. PUFA; see section 4.3). C22:5*cis* caused problems in the full model and was therefore removed. The SAS procedures set the coefficient of C22:5*cis* to zero, as it seemed to be a linear combination of various other variables (including dietary variables), which led to a model with less than full rank and biased estimates.

These considerations led to Pool1 (section 3.6) which contained the 62 potential predictors for EB. Pool2, which was also used in stepwise model selection, contained individual FA, exclusively, except for C22:5*cis*.

4.3 Pearson Correlations between Potential Predictor Variables and Energy Balance

In Table 12 the Pearson correlations of EB with the potential predictor variables and the variables which were actually used to calculate EB are displayed. The correlations greater than 0.125 in absolute value ($|r| > 0.125$) were significant at the 5%-level. Correlations were mainly low (0.0–0.3) to moderate (0.4–0.6). The correlation between EB and DIM of 0.55 was among the strongest and positive, as expected. The correlations between EB and MYs (-0.46) and EB and DMI (0.50) were comparable in magnitude but opposite in sign. Milk fat was negatively correlated with EB (-0.21), while milk protein was positively correlated with EB (0.40). These findings are roughly in accordance with Grieve et al. (1986) who reported significant correlations between milk fat and EB (-0.33 to -0.65) for 6 of 10 dietary treatments and between milk protein and EB (0.34 to 0.47) for 5 of 10 dietary treatments.

Table 12 Pearson correlation coefficients (r) of energy balance (EB) with potential predictor variables (correlations greater than 0.125 in absolute value, i.e. |r|>0.125, are significant at the 5%-level; variables preceded by # were not used for EB prediction)

Variable	r	Variable	r	Variable	r
Diet composition		C13:0*iso*	0.33	C18:3c9,c12,c15	-0.28
NEL (MJ/kg DM)	0.16	C13:0*anteiso*	0.47	C19:0	n.s.
OM (g/kg DM)	-0.14	C14:0	0.62	C20:0	n.s.
CP (g/kg DM)	-0.08	C14:1*cis*	0.38	C20:1c9	0.27
uCP (g/kg DM)	-0.16	C14:0*iso*	0.28	C20:1c11	-0.38
RNB (g/kg DM)	n.s.	C15:0	0.51	C20:3*n-6*	0.39
CF (g/kg DM)	n.s.	C15:0*iso*	0.45	C20:4*n-6*	n.s.
CL (g/kg DM)	-0.19	C15:0*anteiso*	0.46	C20:5*n-3*	-0.23
Animal performance		C16:0	0.46	C22:0	n.s.
Parity	n.s.	C16:1*trans*	n.s.	#C22:5*cis*	n.s.
DIM (d)	0.55	C16:1*cis*	-0.28	#SFA	0.59
#BW (kg)	n.s.	C16:0*iso*	0.25	#UFA	-0.59
#DMI (kg/d)	0.50	C17:0	n.s.	#MUFA	-0.61
#MYw (kg/d)	-0.46	C17:1	n.s.	#PUFA	n.s.
MYs (kg/d)	-0.49	C17:0*iso*	n.s.	#SCFA	0.26
#ECM (kg/d)	-0.52	C17:0*anteiso*	-0.17	#MCFA	0.60
Milk composition		C18:0	-0.33	#LCFA	-0.59
Milk protein (%)	0.40	C18:0*iso*	-0.55	#OCFA	0.45
Milk fat (%)	-0.21	C18:1t6-t11	-0.13	#BCFA	0.20
Milk lactose (%)	n.s.	C18:1t12-t14	n.s.	#∑C16	0.46
#Urea (mg/dl)	0.31	C18:1c9	-0.62	#∑C18:1*cis*	-0.63
#FPR	-0.42	C18:1c11	-0.55	#∑C18:1*trans*	-0.13
Milk FA (g/100 g FA)		C18:1c12	-0.26	#∑C18:2	n.s.
C4:0	-0.30	C18:1c13+c14+t16	-0.40	#∑*trans*	n.s.
C6:0	n.s.	C18:2t,t-NMID	0.22	#∑C6-15	0.60
C8:0	0.27	C18:2c9,t11	n.s.	#∑*n-3*	-0.29
C10:0	0.41	C18:2c9,t13+t8,c12	n.s.	#∑*n-6*	n.s.
C10:1	0.35	C18:2c9,t12+t8,c13	n.s.	*n-6/n-3*	0.33
C12:0	0.55	C18:2t11,c15+t9,c12	-0.15	C6-15/LCFA	0.60
C12:1*cis*+C13:0	0.54	C18:2c9,c12	n.s.	C15/C17	0.61

NEL = net energy for lactation; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; RNB = ruminal N-balance; CF = crude fiber; CL = crude fat; DIM = days in milk; BW = body weight; DMI = dry matter intake; MYs = milk yield at day of sampling; MYw = milk yield, week's mean; ECM = energy-corrected milk yield (4.0% fat, 3.4% protein); FPR = milk fat-to-protein ratio; FA = fatty acid; c = cis; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; n-3 = *omega-3* FA; n-6 = *omega-6* FA; SFA = saturated FA; UFA = unsaturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SCFA = short-chain FA (<C12); MCFA = medium-chain FA (C12–C16); LCFA = long-chain FA(>C16); OCFA = odd-chain FA; BCFA = branched-chain FA

Generally, the milk FA correlated with EB well in line with theoretical expectations: *de novo* synthesized FA and palmitic acid were positively and relatively strongly correlated with EB with values as high as 0.62 for myristic acid (C14:0), except for butyric acid, which was negatively correlated with EB. This is in accordance with the theory that the mammary *de novo* synthesis

of FA is inhibited by preformed FA during NEB, which leads to a decreased utilization of butyric acid as a primer for milk FA and thus increasing milk butyric acid concentrations (Palmquist et al., 1993). The individual MUFA with chain lengths greater than 14 carbons were mostly negatively correlated with EB, with OA showing the strongest correlation (-0.62) of all the variables. This is consistent with the findings of Gross et al. (2011a) who reported a correlation of 0.77 between NEB and OA based on their investigation of milk FA profile during natural NEB in early lactation and induced NEB in mid lactation. In contrast to this study Gross et al. (2011a) did not use single milk samples but observed the changes in FA profile during a defined time span under constant feeding conditions. Consequently, their data can be assumed to be more accurate than the data in the present study in describing the relationship of EB to milk FA profile. Considering this fact, the similarity of their correlation coefficient with the data in the present study is remarkable and underlines the strong impact EB has on OA concentration in milk. It is not fully clear but it would seem that Gross et al. (2011a) took only the NEB into account concerning its relation to milk FA. This might also partly explain the stronger correlation between EB and OA than in the present dataset, where the whole spectrum of EB was involved: The reaction of milk FA composition to EB should be far more pronounced when EB is negative than when it is positive. If the cow's metabolism is not reacting to energy deficiency by lipomobilization and the mammary gland is sufficiently supplied with energy and substrates for milk fat synthesis under constant and standard feeding conditions, then there should be no significant difference in milk FA profile whether the cow's EB is 10 or 50 MJ NEL in surplus.

This theory is supported by Gross et al. (2011a), who showed that most milk FA did not change significantly as soon as positive EB was reached. Even stronger support is given by the present data shown in Annex 4 displaying the Pearson correlation coefficients between EB and single FA values calculated separately for observations corresponding to negative and positive EB values. Generally, more than twice as many of the 47 single FA were significantly correlated with NEB (27) than with positive EB (12). Correlations were also stronger for NEB, ranging from -0.55 to 0.55, while the correlations of FA with positive EB were relatively weak, ranging from -0.24 to 0.28. Many FA which showed rather strong relationships with NEB, for example OA, C10:0, C12:0, and C15:0, were not significantly correlated with positive EB at all. It can thus be assumed that NEB has a far stronger impact on milk FA profile than positive EB. Consequently, it is tempting to conclude that considering the whole spectrum of EB together could dilute its effect on FA profile. Yet, it was necessary to include positive EB data in modeling to be able to distinguish between negative and positive EB. On the other hand, some FA which showed relatively strong correlations with NEB and no significant correlations with positive EB values had similar and sometimes even slightly stronger correlations with EB when the whole dataset was considered (Annex 4). This means that considering positive EB values

does not necessarily weaken but may even enhance the relationship between EB and some milk FA. Nevertheless, it is important to keep in mind that the correlations between EB and milk FA do not necessarily reflect a direct impact of EB on the FA profile (spurious correlations), especially if FA are very variable and have low concentrations or if individual FA are highly correlated within each other. It may happen that EB has a direct impact on one FA which is in turn highly correlated with another FA for other reasons. This again might result in a high correlation between EB and the second FA, even though there is no direct physiological connection. Therefore, these correlations can only give hints and are not a proof of the direct impact of EB on FA, which would require further investigation to establish.

Gross et al. (2011a) and Ducháček et al. (2012) also concluded that changes in some groups of FA, i.e. SFA, MUFA, *de novo* synthesized (<C16), and preformed FA (>C16) were practicable indicators of changes in EB, besides OA. But these indicators, in their absolute value, could not be combined in a single model for predicting EB because most of them virtually provide the same information: MUFA and LCFA (comparable with preformed FA) mainly consist of OA, and therefore showed high correlations with OA concentration in the present dataset (0.96 and 0.92, respectively, (Annex 3). The group of SFA was highly and negatively correlated with MUFA (-0.99) and thus also with OA (-0.94) because, except for the small amount of PUFA (<4% of total FA), each FA either belongs to the group of SFA or MUFA. This results in a simultaneous decrease in one group if the concentration of the other increases and vice versa, if expressed as a percentage or in g/100 g FA. Rough groupings of milk FA, as used by Gross et al. (2011a), might also combine FA with opposing relations to EB and thus weaken the quality of the FA as predictor for EB. For example, the group of *de novo* synthesized FA contained butyric acid which was negatively correlated with EB – in contrast to the remaining FA. This put the usefulness of the default FA groups for predicting EB into question.

The individual PUFA were either not correlated at all or had very weak correlations with EB, except for C20:3*n-6* which had a moderate correlation of 0.39. As the individual PUFA had low and partly opposite correlations with EB, it was not surprising that the sum of the PUFA resulted in a correlation very close to zero. As this occurred in many FA groups, it can be concluded that using the default FA groups might, in some cases, lead to a loss of information due to the aggregation of FA with opposite relations to EB.

The ratio of C15 to C17 FA (C15/C17) in milk is approximately 2:1, while it is 1:2 to 1:3 in bovine adipose tissue (Raes et al., 2004). Craninx et al. (2008) suggested that C17 FA were preferentially incorporated into adipose tissue and thus must be released in higher amounts during lipid mobilization in times of NEB compared with C15 FA because C17 FA showed a similar curve of concentration in the course of lactation like LCFA. Based on these

observations, Holstermann (2012) proposed that the ratio of C15 to C17 FA could serve as an indicator for NEB. According to this theory, C17 FA should be negatively correlated with EB, as their concentrations are supposed to increase with increasing lipomobilization. This should lower the C15/C17 ratio and lead to a positive correlation of C15/C17 to EB. Indeed, the correlation of C15/C17 to EB was positive and had a value of 0.61, one of the highest correlations. Yet, in view of the present results, this correlation could not be ascribed to the C17 FA, which were not significantly correlated with EB, except for C17*aiso*, but this correlation was very low (-0.17). In contrast, the correlations of C15 FA with EB, ranging from 0.46 to 0.51, suggest that the C15 FA are more likely to be the influential variables in causing the C15/C17 effect. A possible explanation might be a connection between the concentrates supply and the C15 synthesis. A decreased forage-to-concentrate ratio increases the dietary energy concentration and thus contributes to EB in a positive way. Simultaneously, a higher share of concentrates, and thus starch, in the diet results in enhanced ruminal propionate production (Ashes et al., 1997), which is the main precursor for C15 FA synthesis in both mammary and microbial *de novo* synthesis (Jenkins, 1993; Palmquist, 2006). Consequently, increased C15 FA concentrations in milk might reflect increased energy supply by higher amounts of concentrates in the diet and thus have a positive relation to EB, which possibly makes C15 FA alone a more suitable indicator.

4.4 Prediction of Energy Balance

4.4.1 Modeling with Stepwise Variable Selection

The large number of candidate variables for predicting EB, their potentially high pairwise correlations, and complex patterns of interactions made manual selection of the best supported subsets of variables impractical. Additionally, variables that have potentially important information for predicting EB, aside from the physiologically obvious ones like OA, were to be identified. Stepwise selection offers easy automatic variable selection according to pre-specified conditions and was thus used as the first method.

However, these results need to be treated with caution because stepwise selection, though very popular for a time, has been criticized sharply in the past: “[…] if this procedure had just been proposed as a statistical method, it would most likely be rejected because it violates every principle of statistical estimation and hypothesis testing” (Harrell, 2001). The variable selection according to the p-value is problematic as the F-statistics are not F-distributed (Draper et al., 1971) and p-values are too small, while regression coefficients are often too large in absolute value (Harrell, 2001). In the case of collinear variables, which are strongly represented in the present dataset (Annex 3), stepwise selection chooses variables

haphazardly (Harrell, 2001). Derksen and Keselman (1992) reported that the number of candidate variables provided affected the number of noise variables without any predictive value which found their way into the model. The sample size seemed to be of no importance concerning the number of predictor variables in the final model, which can lead to overfitting. Additionally, the quality of the models very much depends upon the selection settings. There is a broad variety of criteria to choose from for a variable, to choose the final model, stop choosing, remove variables, et cetera, which markedly influences the outcome. Hence, modeling with stepwise selection must be handled with care and is according to the criticism not sufficient for identifying important predictors as noise variables are often included, if the model is not pre-specified (Harrell, 2001).

Models obtained by using stepwise selection had reasonable fit statistics (Table 13), with the correlation between the observed and the predicted EB values ranging from 0.82 to 0.96, regardless of whether the predictors came from the whole variable pool (models GLMs-N and -H) or from FA only (models GLMs-FA-N and GLMs-FA-H). Inclusion of interactions in the models led to an improvement of the fit statistics for model GLMs-H but also to a much larger number of candidate predictors (k) than for GLMs-N (60 vs. 21). Using only FA for prediction (GLMs-FA-N) resulted in a reduction in the value of the correlation between observed and predicted values compared with GLMs-N, nevertheless indicating that milk FA can provide substantial information on EB. In order to avoid overfitting, Harrell (2001) recommended that the number of predictors in a model should preferably not exceed 10%, or better, 5% of the sample size, which would be 24 or 12 for n = 248 in the present case. Only for models GLMs-N and GLMs-FA-N the numbers of selected predictors were below 10% of sample size. GLMs-H and GLMs-FA-H exceeded the recommended number of predictors and thus are likely to be over-fitted. That coefficients can be extremely large with stepwise selection, as stated by Harrell (2001), holds true at least for the models including interactions, GLMs-H and GLMs-FA-H, which have coefficients exceeding values of 1.000 up to 18.000 in absolute value (Annex 10). The magnitude of coefficients itself is not the real problem, as it generally not only depends on the importance of a predictor but also on its unit or scale. However, if coefficients of such magnitude appear in a model combined with very small and variable predictors (e.g. C20:1c11 in model GLMs-H), this might lead to a disproportionately large and unstable impact of these predictors on the result. In combination with the highly probable overfitting, this led to the decision that the models GLMs-H and GLMs-FA-H are not ideal for the purposes of this study and are not likely to perform well with independent datasets. Thus, they were excluded from the following modeling steps.

Table 13 Fit statistics for models obtained from stepwise selection (GLMs) with all potential predictor variables and fatty acids (FA) only, with (H) or without (N) interactions and the pre-specified Model MODELpre

Method	Model	k	AICC	r	R^2	R^2_{adj}	RMSE (MJ NEL/d)
GLMs	N	21	2008	0.88	0.78	0.76	12.52
GLMs	H	60	1876	0.96	0.92	0.89	7.60
GLMs	FA-N	14	2089	0.82	0.67	0.65	15.23
GLMs	FA-H	31	2069	0.86	0.74	0.71	13.43

k = number of predictors; AICC = corrected Akaike Information Criterion; r = Pearson correlation between predicted and observed values; R^2 = coefficient of determination; R^2_{adj} = adjusted coefficient of determination; RMSE = root mean square error of prediction; N = only main effects included in model; H = Strong hierarchy imposed, meaning that interactions are included in the model only if all the associated main effects have already been included.

4.4.2 Performance of Regularized Linear Regression Models and Random Forests

As the performance of stepwise selection may be adversely affected by the aforementioned problems, other methods for variable selection and prediction were chosen for comparison. The performance of the regularized linear regression methods, particularly Ridge regression, Lasso, AdaLasso, ENET, ADAENET, and of random forests implemented within a 5-fold cross-validation framework is shown in Table 14. The 5-fold cross-validation resulted in five different models for each method, one for each validation set (see Annex 6 for accuracy of the single validation subsets). The mean accuracy of these models was very similar among all approaches, with the correlation between the predicted and the observed EB values varying between 0.80 and 0.82. The minimum and maximum values of the correlation coefficient were also similar, and the minimum values arose from the same validation set for all methods. However, the number of the chosen predictors varied considerably between and within the different methods. Ridge regression does not perform variable selection in the proper sense but minimizes the coefficients of “irrelevant” predictors and therefore kept all the 62 candidate predictor variables. Lasso and ENET chose 17 to 33 and 18 to 35 effects, respectively, whilst their modified versions AdaLasso and ADAENET selected more intensely and chose 7 to 23 and 6 to 10 effects, respectively.

Random forests also achieved a predictive accuracy of 0.80, taking not only linear relationships into account but also interactions and non-linear relationships. Random forests, being an ensemble method, give no information about the functional relationship between EB and the predictors. However, random forests provide an importance plot which was used to select the 30 most important variables for predicting EB (Annex 5). It served as a kind of a reference method, which demonstrates which predictive accuracy is possible to achieve with these data, when interactions and non-linear relationships are also taken into consideration.

Table 14 Mean accuracy of predictions of the regularized linear regression models and random forests from 5-fold cross-validation

	r			RMSE	Range
Method	Mean	Min	Max	(MJ NEL/d)	of k
Ridge	0.81	0.74	0.84	15.05	62
Lasso	0.81	0.73	0.85	15.09	17–33
ENET	0.82	0.75	0.85	15.16	18–35
AdaLasso	0.81	0.75	0.84	15.14	7–23
ADAENET	0.80	0.74	0.84	17.17	6–10
Random forests	0.80			16.02	62 (30)

r = Pearson correlation coefficient between the predicted and observed EB values; Min = minimum value; Max = maximum value; RMSE = root mean square error of prediction; k = number of predictors within the five validation sets; Ridge = ridge regression; ENET = elastic net; AdaLasso = adaptive lasso; ADAENET = adaptive elastic net.

4.4.3 Reduction of the Combined Regularized Linear Regression Models

The application of the regularized linear regression models and random forests provided a preselection of promising variables from the overall pool of 62 potential predictors. This was an important and useful step in variable selection but it is worth emphasizing that the main model development was conducted using classical methods and required much manual work. The process described in the following is shown graphically in Figure 5.

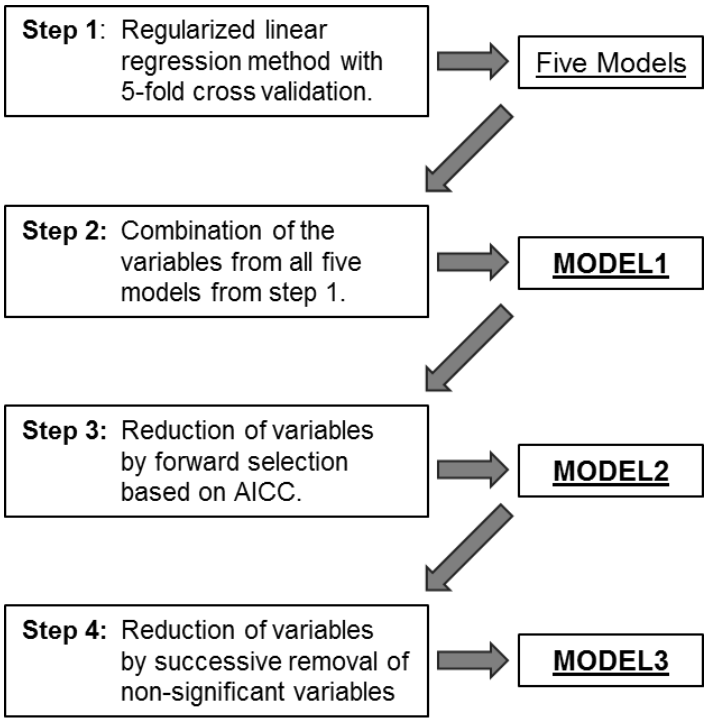

Figure 5 Scheme of the process of modelling with the regularized linear regression models

All variables from the five models of the regularized linear regression methods and random forests, which were selected during the 5-fold cross-validation, were merged into one model for each method (MODEL1), respectively. In the case of random forests, the 30 most important variables according to the importance plot were chosen. This resulted in the full model and five versions of MODEL1 containing 62, 39, 41, 27, 15, and 30 predictors for the Ridge, Lasso, ENET, AdaLasso, ADAENET, and random forests, respectively (Table 15).

Table 15 Fit statistics for the combined models (MODEL1), AICC-reduced models (MODEL2) and p-value-reduced models (MODEL3/4) obtained from different selection methods

Original method	Model	k	AICC	r	R^2	R^2_{adj}	RMSE (MJ NEL/d)
Full Model		62	2096	0.90	0.81	0.74	11.66
Lasso	MODEL1	39	2053	0.88	0.78	0.74	12.44
	MODEL2	13	2036	0.86	0.73	0.72	13.76
	MODEL3	9	2033	0.85	0.73	0.71	13.92
ENET	MODEL1	41	2053	0.89	0.79	0.74	12.31
	MODEL2	14	2041	0.85	0.73	0.71	13.82
	MODEL3	8	2035	0.85	0.72	0.71	14.03
AdaLasso	MODEL1	27	2024	0.88	0.78	0.75	12.55
	MODEL2	11	2035	0.85	0.73	0.72	13.86
	MODEL3	9	2035	0.85	0.72	0.71	13.98
	MODEL4	18	2013	0.88	0.77	0.75	12.83
ADAENET	MODEL1	15	2042	0.85	0.73	0.71	13.80
	MODEL2	11	2044	0.85	0.72	0.71	14.10
	MODEL3	6	2041	0.84	0.71	0.70	14.34
Random forests	MODEL1	30	2069	0.86	0.74	0.71	13.51
	MODEL2	12	2049	0.85	0.72	0.70	14.18
	MODEL3	9	2048	0.84	0.71	0.70	14.35

k = number of predictors; AICC = corrected Akaike Information Criterion; r = Pearson correlation coefficient between the predicted and observed values; R^2 = coefficient of determination; R^2_{adj} = adjusted coefficient of determination; RMSE = root mean square error of prediction; ENET = elastic net; AdaLasso = adaptive lasso; ADAENET = adaptive elastic net

The best supported predictors of EB were then selected using AICC, resulting in MODEL2, with improved AICC for all the methods except for the AdaLasso compared with MODEL1. The correlation between the predicted and the observed EB was 0.01 to 0.04 smaller for all the MODEL2 than MODEL1, a decrease also mirrored by R^2_{adj}, whilst the RMSE increased correspondingly. However, the number of selected predictors also decreased considerably. Thus, with regard to potential practical application, the slight loss of accuracy might be an acceptable price to pay for the enhanced simplicity of the models. The MODEL2s included 11 to 14 predictors. Following this, non-significant effects were successively removed from all the MODEL2, resulting in MODEL3. This step showed even less marked effects on r, R^2_{adj}, and

RMSE. The models could be further simplified to 6 to 9 predictors, without a substantial deterioration in model fit. For AdaLasso the step from MODEL1 to MODEL2 and MODEL3 brought no improvement, therefore, the non-significant effects were directly removed from MODEL1 which led to MODEL4, the best performing model according to AICC.

4.4.4 Pre-specified Model

Retrospectively, a pre-specified model based on physiological and practical considerations was developed. This model included four variables only. First of all, MYs for its estimated high impact on EB and its easy accessibility, and FPR because of its practicability in milk recording, were included. The milk FA component of the model was decided to be the ratio of OA and the sum of the straight-chain SFA C6:0, C8:0, C10:0, C12:0, C14:0, and C16:0 (OA/*de novo*). This index was supposed to combine the impact of EB on body fat mobilization and on mammary *de novo* synthesis of FA. The effect of EB on body fat mobilization (negative correlation) is represented by OA as one of the predominant FA in body fat, which is released into the blood and taken up by the mammary gland in times of NEB (Palmquist et al., 1993). On the other hand, the increased uptake of LCFA from blood causes a decline in the *de novo* synthesis (Palmquist et al., 1993) which is represented by the sum of all straight-chain SFA from C6:0 to C16:0. In positive EB the concentration of the *de novo* synthesized milk FA is higher than in NEB (positive correlation). The resulting ratio was negatively correlated by -0.62 with EB. The fourth variable is the ratio between *n-6* and *n-3* FA (*n-6/n-3*). This decision was made given the obvious high relevance of this variable in most other models, which is discussed later (section 4.4.6). The fit statistics of MODELpre (Table 16) are slightly worse but comparable with the ones obtained with ADAENET and random forests MODEL3.

Table 16 Fit statistics for the pre-specified Model MODELpre

Method	Model	k	AICC	r	R^2	R^2_{adj}	RMSE (MJ NEL/d)
Manual	MODELpre	4	2071	0.82	0.67	0.66	15.37

k = number of predictors; AICC = corrected Akaike Information Criterion; r = Pearson correlation coefficient between predicted and observed values; R^2 = coefficient of determination; R^2_{adj} = adjusted coefficient of determination; RMSE = root mean square error of prediction.

4.4.5 Cross-Validation of the Final Models

The models with the smallest AICC were chosen from each method (Table 15) for the final assessment of model fit using the leave-one-out cross-validation, the results of which are shown in descending order of the correlation between the predicted the observed EB for each model in Table 17. Apart from MODEL4 from AdaLasso the best models were all MODEL3.

The models originating from the stepwise variable selection and the full model were also included in the leave-one-out cross-validation.

Table 17 Fit statistics of the leave-one-out cross-validation for the AICC-selected best models originating from the different selection methods

Original method	Model	k	r	R^2	R^2_{adj}	RMSE (MJ NEL/d)
GLMs	N	21	0.86	0.74	0.71	13.69
AdaLasso	MODEL4	18	0.85	0.73	0.71	13.90
Lasso	MODEL3	9	0.84	0.70	0.69	14.53
ENET	MODEL3	8	0.84	0.70	0.69	14.58
ADAENET	MODEL3	6	0.83	0.69	0.68	14.77
Random forests	MODEL3	9	0.83	0.68	0.67	14.95
-	MODELpre	4	0.81	0.65	0.65	15.68
-	Full model	62	0.81	0.65	0.54	15.96
GLMs	FA-N	14	0.79	0.67	0.51	16.15

k = number of predictors; r = Pearson correlation between predicted and observed EB values; R^2 = coefficient of determination; R^2_{adj} = adjusted coefficient of determination; RMSE = root mean square error of prediction; GLMs = stepwise selection; N = only main effects included in model; FA-N = only milk fatty acids and main effects included; AdaLasso = adaptive lasso; ENET = elastic net; ADAENET = adaptive elastic net; MODELpre = pre-specified model

According to the correlation between the predicted and the observed EB values, the GLMs-N model performed the best, followed by models selected by the AdaLasso, Lasso, the ENET, ADAENET, and random forests, all of which differed only marginally in terms of their performance ranking in cross-validation. The performance of the GLMs-FA-N model was the worst followed by the full model. The drop in prediction accuracy resulting from model development (Table 13 and Table 15) versus leave-one-out cross-validation (Table 17) was virtually negligible (r = 0.01 to 0.04), except for the full model (r = 0.09). However, the last two models, the full model and GLMs-FA-N, also showed strong declines in R^2_{adj} of 0.20 and 0.14, respectively, while the declines for the other models were within the range of 0.02 to 0.05. In conclusion, the most stable performance was shown by the models based on the regularized linear regression methods and the differences in accuracy between them are negligible.

The full model performed rather poorly in cross-validation, showing that it overfitted the present data and thus can be expected to perform poorly with future independent datasets. This is not surprising because a model such as the full model containing far more predictors than recommended relative to the sample size (25%) and many non-significant predictors (30 of 62; Annex 7) can be expected to overfit the data and perform poorly when applied to independent datasets (Burnham and Anderson, 2002).

The model containing only FA (FA-N) performed worse than the others and showed a great decline in accuracy in cross-validation. This, again, is not surprising as very informative variables like MYs and DIM were not available. Still, the amount and nature of the information provided is interesting from the physiological point of view as this model does not contain any variables which are directly connected to the calculation of EB (i.e. MY, milk fat, and protein). It gives information about the amount of variation in EB which can be explained only by milk FA (R^2 = 0.62 in leave-one-out cross-validation). With regard to the complexity of milk fat composition and the fact that single milk samples were taken from cows of six different farms being fed 13 different diets in contrast to most other studies with similar aims (Kay et al., 2005; Gross et al., 2011a; Ducháček et al., 2012; Nogalski et al., 2012), this is a remarkable result.

As the predictions are not very precise, problems can occur, particularly along the borderline of positive EB to NEB. If EB is predicted in the positive range, but is negative in truth, this is called a false positive prediction. Vice versa, when EB is predicted as negative but is positive in truth, this is called a false negative prediction. If this happens, it might cause misleading conclusions. Thus, although the EB predictions are continuous, in practice the most important information these models could provide would be of a binary kind: is the EB positive or negative? Or, how high is the risk of false negative or false positive predictions?

During leave-one-out cross-validation each model is fitted 248 times, each time leaving out only one observation whose value is to be predicted. The raw deviations are the differences (prediction errors) between the predicted and the observed values obtained during leave-one-out cross-validation. They provide a quantitative measure that can be used to minimize such misclassifications. The means of these raw deviations were close to zero for all models as expected (-0.05 to 0.04 MJ NEL/d), the standard deviation of the raw deviations (SD_{rd}) ranged from 13.72 to 16.19 MJ NEL/d (Table 18). Figure 6 illustrates the raw deviations of model GLMs-N for each observation. The lowest overall SD_{rd} was 13.72 MJ NEL/d. One could thus assume that the predictions of this model are largely correct within a ±13.72 MJ NEL/d error margin. Considering this, one could further speculate that only predicted EB values below or above one SD_{rd} (±13.72 MJ NEL/d) are probably truly negative or positive. This suggestion seems to be confirmed by the data in Table 18, in which EB values with predictions falling in the wrong range (false positive/negative) are displayed. The mean percentage of false positive or false negative predictions ranged from 14.5 to 22.2%. If only predictions falling outside the range of the SD_{rd} of the prediction error are considered, the number of falsely predicted values decreased strongly, as presumed, to 1.2 to 2.4% of total observations. The fact that there are markedly less false positive than false negative predictions beyond the range of SD_{rd} supports the hypothesis that NEB might be easier to predict than positive EB. Here, the best performance is shown by the ADANET and the random forests derived models.

Table 18 Number and percentage of false positive and false negative predicted values in the total dataset and beyond one standard deviation of the raw deviations (SD_{rd} in MJ NEL/d) from the leave-one-out cross-validation for each final model

			complete dataset (n = 248 samples)			beyond range of SD_{rd}		
Original method	Model	SD_{rd}	false positive	false negative	total %	false positive	false negative	total %
GLMs	N	±13.72	19	18	14.9	1	4	2.0
AdaLasso	MODEL4	±13.93	17	20	14.9	1	4	2.0
Lasso	MODEL3	±14.56	18	21	15.7	1	3	1.6
ENET	MODEL3	±14.60	22	25	19.0	1	3	1.6
ADAENET	MODEL3	±14.79	22	21	17.3	0	3	1.2
Random forests	MODEL3	±14.98	17	22	15.7	0	3	1.2
-	MODELpre	±15.72	24	31	22.2	2	3	2.0
GLMs	FA-N	±16.19	23	17	16.1	2	4	2.4

ENET = elastic net; AdaLasso = adaptive lasso; ADAENET = adaptive elastic net; GLMs = stepwise selection; N = only main effects included in model; FA-N = only milk fatty acids and main effects included; MODELpre = pre-specified model

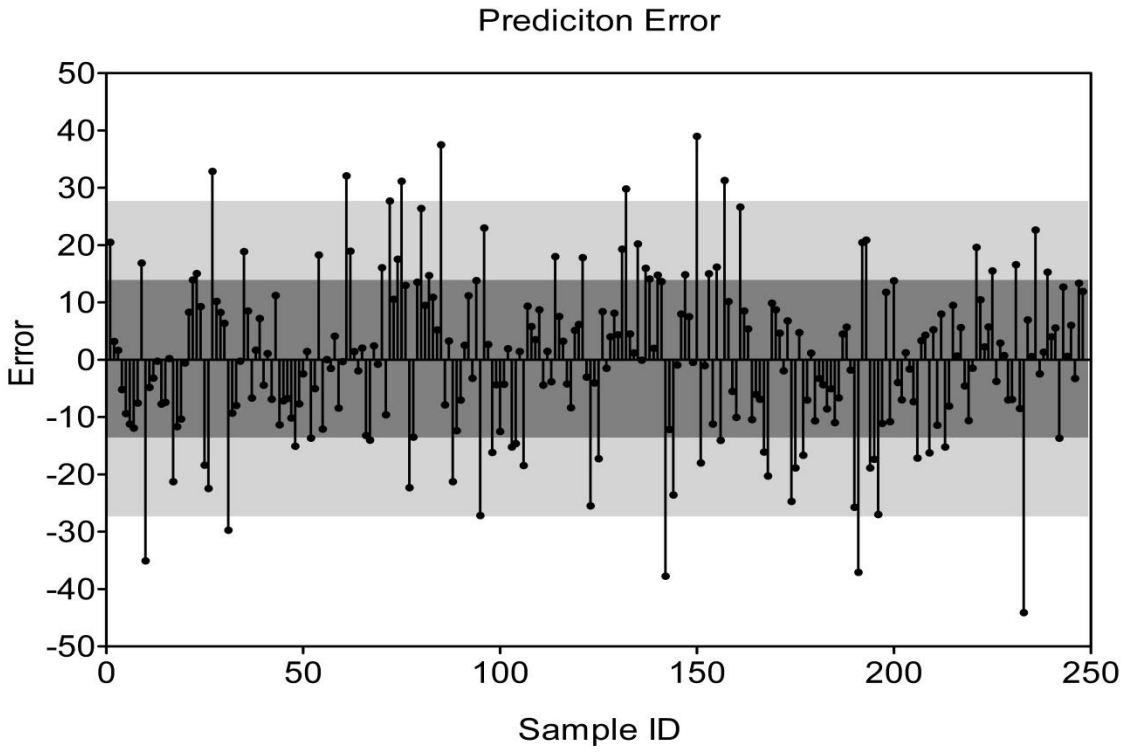

Figure 6 Raw deviations (black dots) of the predicted from the observed EB values (MJ NEL/d) for each observation (n = 248 samples) obtained from leave-one-out cross-validation of model GLMs-N. The band shaded deep grey marks one standard deviation (±13.72 MJ NEL/d) whereas the band shaded light grey marks two standard deviations.

A consequence of this consideration is that a total EB range of approximately 28 to 32 MJ NEL/d (from approximately -14–16 to +14–16 MJ NEL/d) is hard to predict with adequate reliability. In the present dataset this applies to 100 to 152 observations which constitute 40 to 60% of the total of 248 observations. For scientific purposes this level of accuracy would be not considered satisfactory. However, for practical purposes it might be acceptable as the cow can tolerate a certain energy loss for some time without her health or performance being compromised (Butler and Smith, 1989). Chalupa and Harrison (1996) suggested that early lactating cows can tolerate an energy deficit of approximately 10 to 15 MJ NEL/d for about three weeks. This suggestion perfectly fits the present models as it is within the range of the SD_{rd} of most models. And also for the positive range of EB, this might be suitable because after the peak MY the cow needs a certain amount of surplus energy to replenish her body reserves, but in order to avoid obesity the energy supply also should not be too excessive, which could probably be detected by the models.

Altogether, the models derived from the variables constituting Pool1 showed that only a few variables (6 to 9) are necessary to achieve good predictions of EB. However, some models containing more variables (GLMs-N, AdaLasso MODEL3) yielded better accuracy, indicating that there are many variables each of which contributes a small amount of additional information. The model GLMs-N outperformed the other models in terms of accuracy, even after cross-validation, and also had unstandardized and standardized coefficients similar to those of the other models, with common selected variables. Therefore, it can be concluded that this method is also worth being considered for prediction purposes.

4.4.6 Standardized Coefficients of the Final Models

Table 19 shows the standardized coefficients of the best models for each selection method. This allows evaluation of the relative contribution of each predictor to predicted EB independently of its unit of measurement or degree of concentration in milk. The variables which occurred in all models derived from Pool1 were DIM, MYs, C15:0*iso*, OA, and *n-6/n-3*. The tables Annex 8 and Annex 9 show the unstandardized coefficients of the final models.

As expected, the effects MYs and OA have large standardized coefficients compared with the others within each model, and thus can be regarded as very important. Feed composition variables did not appear in any of the final models, except for the ruminal nitrogen balance (RNB) in AdaLasso MODEL4 and GLMs-N, suggesting that the information they contribute can be substituted or is contributed by the other variables. As it was a component of the EB calculation, energy content of the diet was expected to play a role in the models but it did not. This might be a consequence of the very low variation in the energy content of the diets, which

might also apply to the other feed variables. The apparently high importance of C15:0*iso* and *n-6/n-3* was unexpected and merits a closer look:

The main component of the *n-6* FA is LA, while the *n-3* FA mainly consist of ALA. As stated before (section 2.3.2), the grain content of the diet has a strong impact on the *n-6/n-3* ratio. If the forage-to-concentrate ratio decreases, the concentration of ALA decreases, while LA can increase, and thus, the *n-6/n-3* ratio also increases (Patel et al., 2013). Following the same principle, the *n-6/n-3* ratio increases, if grass silage is replaced by corn silage because of its higher proportion of grains which leads to decreased intake of ALA and increased intake of LA (Chilliard et al., 2001b; Ferlay et al., 2006; van Gastelen et al., 2015). Increasing amounts of corn silage and/or concentrates increase the energy content of the diet und thus contribute positively to EB, which might explain the positive correlation between EB and *n-6/n-3*. This correlation was found to be weak but significant (0.33; Table 12). However, milk LA itself is not correlated with EB, which might be a consequence of the fact that forages as well as concentrates can be a source of LA, especially corn silage (Harfoot and Hazlewood, 1997). On the other hand, ALA mainly originates from forages, especially grass based ones. It is thus negatively associated with dietary grain content (van Gastelen et al., 2015) and negatively correlated with EB, as expected, if weakly (-0.28). Consequently, ALA must be the main contributor to the correlation of *n-6/n-3* with EB. Physiologically, the connection between EB and *n-6/n-3* can be explained, however, in the present dataset it was not very strong. Using multivariate factor analysis, Conte et al. (2016) investigated the relationship among milk FA and found that ALA was the only FA which was uncorrelated with other FA. In the present dataset ALA is indeed correlated with other variables, but these correlations are low to moderate. The highest correlations of ALA are those between ALA and C18:1*trans* FA (up to 0.54; Annex 3), which might arise from their connection via the ruminal biohydrogenation process. Thus, the importance of this variable in the form of the *n-6/n-3* ratio might arise from the fact that it is largely independent from the other predictors, i.e. there is no appreciable collinearity.

This might imply that the use of FA as predictors, which have low and highly variable concentration and are highly intercorrelated, is not appropriate. Perhaps coincident dietary conditions in the present dataset are partly responsible for these results. If this would be the case, then the models would probably not be applicable to other datasets without further refinements.

Table 19 Standardized coefficients of the final models (FA variables in g/100 g FA, coefficients marked with "*" are not significant at the 5%-level)

Original method	Lasso	ENET	Ada-Lasso	ADA-ENET	Random forests	GLMs	GLMs	-
Effect	MODEL 3	MODEL 3	MODEL 4	MODEL 3	MODEL 3	N	FA-N	pre
Intercept	-6.5	-6.5	-6.5	-6.5	-6.5	-6.5	-6.5	-6.5
DIM (d)	51.8	44.8	57.9	48.6	70.2	52.8		
RNB (g/kg DM)			47.0			57.3		
MYs (kg/d)	-148.1	-146.7	-135.8	-154.5	-131.5	-137.5		-171.3
Milk fat (%)	-67.5	-82.3	-77.6	-76.2		-73.9		
C6:0			-54.2				-70.8	
C8:0	-39.7	-69.9				-80.8		
C12:1*cis*+C13:0			77.4			149.7	147.5	
C14:0*iso*			55.9		46.4	52.8	80.7	
C15:0						-70.1*	-110.8	
C15:0*iso*	125.5	104.6	109.8	99.0	110.1	118.8	180.6	
C16:0							117.5	
C16:1*cis*			50.7			40.9*		
C17:0			-58.4			-51.1		
C18:1c9	-177.4	-185.8	-141.1	-145.9	-119.3	-178.1	-137.8	
C18:1c11	64.9		100.5		90.6	118.7	134.2	
C18:2t,t-NMID						-37.3*		
C18:2c9,t11						42.6		
C18:2c9,t13+t8,c12							91.2	
C18:2c9,c12			80.4			75.8	153.8	
C18:3c9,c12,c15			-71.3			-67.4*	-163.7	
C19:0		-49.2						
C20:0			92.6			99.6	89.3	
C20:1c11	-57.6		-97.5		-52.4	-111.4	-100.1	
C20:5*n-3*							-42.0	
C22:0			-42.6			-43.5		
n-6/*n-3*	141.5	129.6	81.0	131.5	153.3	88.9		98.8
C15/C17					60.8			
OA/*de novo*								-186.3
FPR								-111.2

AdaLasso = adaptive lasso; ENET = elastic net; ADAENET = adaptive elastic net; GLMs = stepwise selection; FA = fatty acid; GLMs-FA = stepwise selection with FA only; N = only main effects included in model; MODELpre = pre-specified model; DIM = days in milk; uCP = utilizable crude protein at the duodenum; RNB = ruminal N-balance; CF = crude fiber; c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA; MYs = milk yield at day of sampling; OA/*de novo* = C18:1c9/(C6:0+C8:0+C10:0+C12:0+C14:0); FPR = milk fat-to-protein ratio

Generally, considerable changes in the OCFA and BCFA profile in milk are associated with shifts in rumen microbial population which in turn predominantly depends on feeding conditions (Vlaeminck, 2004; Vlaeminck et al., 2006a). The *de novo* synthesis of *iso*-FA is exclusively performed by rumen microbes, especially by cellulolytic bacteria. Consequently, comparable with ALA, the milk concentration of C15:0*iso* increases with increasing forage-to-concentrate

ratio or when corn silage is replaced by grass silage as forage component (Patel et al., 2013; Shingfield et al., 2005; Vlaeminck et al., 2006a; Vlaeminck et al., 2006b; Buccioni et al., 2012). Following the same argument as for *n-6/n-3*, C15:0*iso* would thus be expected to be negatively correlated with EB, like ALA. However, the correlation of C15:0*iso* with EB is positive and relatively strong (0.45) and it is the same with all BCFA except for C17 BCFA. Connections between milk FA and EB via dietary composition should be made carefully, the more so because early lactating cows usually receive high energy, high starch diets and are in NEB, anyway. On the other hand, C15:0*iso* as a ruminal derived FA might be related to EB via the DMI. During the observed lactational stage EB increases along with increasing DMI rather than decreasing MYs as shown by the moderate positive correlation of DIM with DMI and EB and the very low negative correlation between DIM and MYs (0.42, 0.55, and -0.22, respectively). Thus, it might be concluded that in early lactation DMI might be the crucial factor determining EB. If a high DMI of a balanced diet improves EB and concurrently the ruminal environment by supporting growth of rumen microbes, and thus microbial FA synthesis, there could be an indirect physiological connection made between C15:0*iso* and EB. Unfortunately, this would also imply a relatively high and positive correlation between DMI and C15:0*iso*. This correlation indeed is positive but very low (0.18). The same might apply for C14:0*iso* which additionally occurs in AdaLasso MODEL4, Random forests MODEL3, GLMs-N, and GLMs-FA-N.

C18:1c11 had positive and large standardized coefficients, despite having a negative correlation with EB (-0.55). This may be a consequence of the suppression of its effect by another predictor with which it is highly correlated (Smith et al., 1992). Indeed, C18:1c11 is highly correlated with OA (0.79), but the content of OA in milk is about 20 times higher than that of C18:1c11. There are several other variables showing the same phenomenon, especially in the models selected by AdaLasso and GLMs-N. However, it may also partly reflect differences in the variances of the estimated effects of the predictors. Other effects with correlations and standardized coefficients with opposite signs were: C8:0, which is positively correlated with EB (0.27) and negatively correlated with OA (-0.61) and has exclusively negative coefficients. C15:0 is highly and positively correlated with C12:1*cis*+C13:0 (0.89) and moderately correlated with EB (0.51), however, its standardized coefficients are negative. These FA appear in the GLMs-N and GLMs-FA-N models. The elastic net-type selection methods were the only methods which led to a final model without suppression effects. However, both models also contained at least one pair of these FA in one of the five cross-validation sets in the initial run. Here, ADAENET was the only method which originally assigned a negative coefficient to C18:1c11. Thus, this method might perform better with highly intercorrelated data than the other selection methods. In contrast to GLMs the regularized linear regression methods used, especially ENET and ADAENET, are intended to be able to

handle highly correlated predictors. Consequently, they should be preferably applied to datasets with highly correlated variables like the present one.

As many milk FA arise from the same pathway of synthesis, it was not surprising that they were correlated with each other. The FA C8:0, C10:0, C12:0, and C14:0 are also linked through their biosynthesis pathway which is reflected in the pattern of their correlations. During FA synthesis catalyzed by fatty acid synthase, two carbons, usually from a malonyl moiety, are added to the primer (i.e. acetyl-CoA or β-hydroxybutyryl-CoA) and then to the growing FA chain with each performed cycle until the FA is released with usually 12 to 16 carbons (section 2.2.3). Each of the mentioned FA is highly and positively correlated (approx. 0.9, Annex 3) to its precursor and its successor, but with each further addition or removal of C2 the correlation declines by approximately 0.2. For instance, the correlations of C6:0 with C8:0, C10:0, C12:0 and C14:0 are 0.91, 0.71, 0.51, and 0.35, respectively. The results of Karijord et al. (1982), investigating the sources of variation in milk FA profile, showed almost the same pattern. On the other hand, Moate et al. (2007) who conducted a meta-analysis of the variation of milk FA concentration including 29 experiments and 120 different dietary treatments, achieved different correlations. While the correlation of C6:0 with C8:0 was similar as above (0.95), the correlations of C6:0 with C10:0 to C14:0 did not decrease but were all in the same range (0.85–0.87). This might be a consequence of the different data structure as many feeding trials included high fat diets, specific fat supplements, and abomasal FA infusions. These treatments substantially manipulate the FA metabolism, which might lead to a different correlation pattern, while the animals in the study of Karijord et al. (1982) were likely fed under practical conditions and their results are therefore more comparable to the ones of the present study.

The positive correlation of the aforementioned FA (C8:0, C10:0, C12:0, and C14:0) with EB increases with each attached C2-complex along this pathway, reflecting the fact that the *de novo* synthesis decreases with decreasing EB and vice versa (Palmquist et al., 1993). Short-chain FA might be more weakly correlated with EB than MCFA because their existence in milk is more or less incidental, as they are usually elongated up to C12–C16 (Palmquist, 2006). This physiological link can explain why there was never more than one of the aforementioned FA in a model – they are too closely related. On the other hand, one could argue that C8:0, which often occurred in the models, is by far less correlated with EB than C12:0 or C14:0. However, these FA are also highly correlated with OA and thus, their incorporation into the models would not likely provide a high amount of additional information which could make C8:0 a better choice. Additionally, according to the contents of minor FA in milk shown by MacGibbon and Taylor (2006), C13:0 accounts for approximately 75% of C12*cis*+C13:0, which are shown as a sum because it was not possible to distinguish them analytically. Thus, it is

plausible, that the highly positive correlation of C15:0 with C12:1*cis*+C13:0 (Annex 3) arises from the fact that C13:0 has the same synthetic route as C15:0 (and then C17:0).

In summary, even if not every single variable is discussed, most of the variables occurring in the models can be physiologically explained. The predictive quality of the models and their composition is generally quite similar, but they can be categorized into two groups: The one group (Lasso, ENET, ADAENET, Random forests derived models and MODELpre) contains a low number of predictors (6–9), which is beneficial in terms of simplicity, practicability and interpretability, but it also has slightly lower predictive values. The other group (AdaLasso, GLMs-N, and GLMs-FA-N), on the other hand, contains more predictors (14–21) and shows better accuracy. Each additional predictor seemed to provide little but significant additional information and thus the sum is likely the reason for the better accuracy.

4.5 Previous Attempts of Modeling or Predicting Energy Balance Compared with the Present Results

Several studies have modelled and analyzed EB in one or more entire lactations using different approaches (de Vries et al., 1999; de Vries and Veerkamp, 2000; Coffey et al., 2002; Banos et al., 2005; Strathe et al., 2010; Strathe et al., 2011; Thorup et al., 2012; Thorup et al., 2013). However, only few studies have focused on predicting EB from milk traits (Heuer et al., 2000; Reist et al., 2002; Friggens et al., 2007; Mäntysaari and Mäntysaari, 2010). These mainly took changes in milk and body traits into account and obtained results comparable with those from the present study. None of the latter studies tried predicting EB from single observations which were not derived from repeated measurements. This complicates proper comparison of their results with the results of the present study.

In a study by Heuer et al. (2000) 72 HF cows were observed for 13 weeks from calving to predict early lactation EB at the individual and herd levels. Milk composition was analyzed four times a week; once a week cows were scored for BCS and blood samples were taken. Cows did not receive the same diet but were divided into six groups receiving different types of concentrates. This approach is the most comparable one to the present study. True EB was calculated traditionally, by balancing energy input and output in a similar way as done in the present study. Week of lactation, parity, MY, and milk composition were used as potential predictor variables. A multivariate linear model was developed by successive addition of variables. The authors created a base model containing week of lactation, parity, and MY, which was extended with milk components in various ways. The model selected for herd level prediction consisted of the base model plus milk protein and FPR. The variation in EB explained by this model was 25.1%. The very best model explained 29.1% of the variation in

EB and consisted of the previous model plus the interaction of week of lactation and treatment. These predictive accuracies are quite low compared with the ones obtained in the subsequently discussed study (Heuer et al., 2001) and also of the present study. The authors argued that the correlations between EB and variables affecting the energy requirement (MY, milk solids) are very small (R^2 = 0.01–0.06, which corresponds to r = 0.10–0.25) and thus, the usually unknown factor of energy intake must have a much greater impact on EB than the requirement. From the perspective of the present study, where the correlation between EB and MYs, and EB and energy intake was 0.46 and 0.50, respectively, this theory cannot be fully confirmed. It might be correct that DMI is the most critical factor in early lactation (see section 4.4.5), however, the correlation between MY and EB in the present study appeared too strong to be meaningless.
Subsequently, Heuer et al. (2001) applied their previously developed model for predicting EB at the herd level (Heuer et al., 2000) to test-day milk records and found it reasonable when standard diets were used.

Similar to the aforementioned study, Reist et al. (2002) also predicted EB at the individual and herd levels on the basis of milk performance and composition, animal data and blood traits of 90 early lactating cows. Cows were either assigned to a group which received 50% concentrates in the diet or 30% on a DM basis. True EB was calculated using the same traditional equation as used in the present study. Body weight and MY were measured twice daily, while milk composition was obtained four times a week. Until week 10 postpartum blood samples were taken and milk acetone was determined on a weekly basis. Mixed models were developed with cow as a random effect, treatment and week of lactation as categorical fixed effects, milk and/or blood traits as continuous covariates. Best results were achieved with a model containing all the aforementioned categorical variables and ECM, milk fat-to-lactose ratio, and plasma NEFA, creatinine, and thyroxine concentrations. R^2 was 0.90 for the complete model (including cow as a random effect) and 0.71 for fixed effects only, which is in the same range as the results of the present study (R^2 = 0.65–0.74, see Table 17). Model precision was ±13.0 MJ NEL/d which is also comparable. Using milk acetone instead of blood traits led to very similar results. This led to the conclusion that blood traits are substitutable, which is clearly beneficial in practice. This study achieved nearly the same results with far less (and easier to obtain) variables than the present one. This might result from the inclusion of the two feeding treatments by Reist et al. (2002), which might have a strong impact on EB, especially for the cows fed only 30% concentrates. Logically, these animals must have had a lower EB than the 50% group, which is also shown by the negative regression coefficients assigned to the 30% group in the respective models. In contrast, in the present study various diets were used and exclusively ones which aimed to meet energy requirements as far as possible in early lactation. However, the authors also argued that the precision of prediction is

too low for most practical purposes. As discussed before, this depends on the demands imposed on the model. It might still be practicable as an early warning system for very low EB.

Using performance data of 146 primiparous Red Dairy Cattle cows Mäntysaari and Mäntysaari (2010) predicted EB from changes in body traits (BCS, BW), their changes from calving to first milk recording and the respective milk recording data. The authors desisted from including MY into the models because of its major role in EB calculation (input-output). The best result was achieved using a linear model which contained FPR, BW, change of BCS from calving to test-day and its interaction with BW. The coefficient of determination of this model accounted for 0.39. The authors discussed imprecision of EB calculation as a factor of this low quality prediction, as they estimated BW from heart girth measurements instead of weighing the animals. As Friggens et al. (2007) did, Mäntysaari and Mäntysaari (2010) identified FPR as the milk trait most strongly related to EB.

Friggens et al. (2007) predicted EB using longitudinal data of two lactations of 299 cows. They calculated the true EB from measures of changes in body reserves, including ΔBW, ΔBCS, and gut fill to avoid using milk traits as common in traditional input-output EB calculation and ensure independence of the predictor variables. In the present study, no longitudinal data were available, thus, calculating EB in the traditional way was unavoidable. As a compromise, the mean MY of the week before sampling (MYw) was used for output calculation and only the MY of the day of sampling (MYs) was used as a predictor variable, which is very closely related, but yet not the same. Friggens et al. (2007) performed the EB prediction using partial least squares regression, also a linear approach, and exclusively milk components, their ratios and differences, and DIM as predictors. On the one hand, they predicted EB using group mean data within breeds and parities, which led to very good results. One model, containing only 5 of the 25 potential predictor variables, explained 94% of the variation of EB with a prediction error of 3.9 MJ/d. On the basis of individual data instead of group means, they achieved poorer results with a prediction error of 17.3 MJ/d and the correlation between the predicted values and the retrospectively calculated traditional EB was 0.70. Since Friggens et al. (2007) used longitudinal data of complete lactations of more cows than total observations were used in the present study, one can expect that their predictions are more accurate and stable than the ones achieved here. Yet, their prediction error using individual data and all available predictors was comparable with the results achieved in the present study.

A companion paper (Løvendahl et al., 2010) to Friggens et al. (2007) claimed that EB calculated from body traits were more accurate than EB predicted from milk traits. This is not surprising as they obviously used the same dataset as Friggens et al. (2007) and compared their EB prediction equation with the EB calculation based on body traits that was used as a response variable for the development of the EB prediction with milk traits.

With respect to these results, it might be concluded that it is possible to predict EB with the help of MY, milk composition and milk FA from single observations with an acceptable accuracy. However, some previous attempts in predicting EB with continuous data and more easily accessible traits than milk FA showed similar model precision (Heuer et al., 2000; Friggens et al., 2007). On the other hand, including milk FA concentrations in EB prediction might provide substantial additional information, which seems able to compensate for the lack of continuous data and therefore Δ-values as predictors. If future technical progress enables farmers to frequently measure FA composition during milking, a combination of both might help to create a powerful management tool for monitoring EB.

4.6 Conclusions

- It is possible to predict EB from milk FA profile using single milk samples from cows of different origin and fed different diets (without particular fat supplementation). The accuracy of the obtained models is comparable with others from similar studies using continuous data and other milk and animal traits than milk FA and the animal traits used here. This suggests that milk FA provide substantial additional information in addition to raw milk components, MY and stage of lactation if no continuous data are available.

- The methods which were used for the initial variable selection from the pool of 62 potential predictors all provided final models which were similar in structure and comparable in accuracy. Although stepwise variable selection is criticized by statisticians from a theoretical standpoint, it provided the best final model and might therefore nevertheless be a sufficient, easy and quick tool for issues of this kind in practice. However, the differences in accuracy between the applied methods were very small. As regularized linear regression methods, especially ENET and ADAENET, are supposed to deal better with highly correlated variables, it might be safer to use them with datasets containing highly correlated variables as the present one does.

- The accuracy of the final models validated using leave-one-out cross-validation did not decrease strongly compared with the results of the previous model fitting. It was shown that not many predictors are needed to obtain good predictions, with a predictive ability (correlation between the observed and predicted EB values) ranging from 0.79 to 0.86. However, the applicability of these models to independent datasets may be limited for the following reasons:
  - the number of observations in the present dataset (n = 248) was relatively low and thus it is possible that the models may not be stable enough to maintain the same level of predictive accuracy with independent datasets, especially because the current data

may not capture enough variation in the feeding conditions. Dietary traits did not enter the models, presumably because of their low variation as the diets fed had similar composition. However, in practice, even without special fat supplementation there can be substantial differences in diet composition able to influence EB or the milk FA profile or both.

- differences in GC analysis methods of the milk FA might spoil the predictions as there are some very low concentration and very variable FA in the models which might be more prone to laboratory effects than FA contained in higher concentrations.

- A practical application of the developed models is not yet possible, even if a good performance with independent datasets would exist. First of all, the costs for GC analysis are far too high for commercial farmers. There are efforts being made to predict milk FA from MIR spectra (Soyeurt et al., 2006a; Soyeurt et al., 2011; Ferrand-Calmels et al., 2014), which would be a cheap alternative, but so far only highly concentrated FA can be predicted with sufficient accuracy. However, applying effects which were predicted themselves on a prediction model may lead to an accumulation of errors and thus impair prediction accuracy. Therefore, the development of an accurate, fast and low-cost analytical method for FA was considered desirable with respect to a possible application of milk FA in EB predictions.

  Additionally, as discussed in section 4.4.5, with respect to the danger of false negative or false positive predictions, the models may at best be useful as a warning system for detecting very high or low EB, beyond approximately ±16 MJ NEL/d. This would result in discarding 40–60% of the predictions, which is very high. Whether the accuracy of the models could be further improved by using a larger dataset is also questionable, as milk FA are highly variable traits influenced not only by EB, but also by feed factors, stage of lactation, breed and other genetic and individual factors, ruminal microbial population (which in turn highly but not exclusively depends on dietary conditions), and other factors.

- The FA profile in the present dataset was well in line with physiological expectations. Some low concentrated and very variable FA (e.g. C15:0*iso*) entered the models and showed significant impacts on the EB prediction. Their physiological connection to EB, if truly present, could not be fully clarified and would require further research in the form of experimental trials under standardized conditions.

5 SUMMARY

In early lactation, when feed intake cannot meet the energetic requirements caused foremost by milk production, dairy cows experience negative energy balance (NEB) to which they react by mobilizing body reserves. This is principally a natural process. However, after decades of breeding cows for higher milk performance, this energy deficit has intensified in a way that it strongly contributes to health problems like ketosis, fatty liver syndrome or impaired fertility. Thus, attempts for monitoring of the energy balance (EB) gained more and more importance; be it for a warning system for cows experiencing very low EB for a too long time-span or for detecting cows which are able to adapt better to the situation than others in order to select them for breeding. However, EB monitoring is not easy to perform without measurement of the individual feed intake which is only possible under conditions of experimental research stations.

The objective of the present study was to predict EB of dairy cows from animal, feed, and milk traits. As the milk fatty acid (FA) profile is known to react to physiological conditions like an energy deficit, special regard was given to milk FA in order to identify new potential indicators for NEB. Visiting six experimental stations in Germany, single milk samples were taken from dairy cows between their 6th and 133th day in milk to create a dataset covering a large spectrum of EB and a variety of practical diets. The milk composition was analyzed by mid-infrared spectrometry, and the milk FA profile via gas chromatography. Energy balance (MJ NEL/d), as response variable, was calculated by subtracting the cow's energy requirements from energy intake. As candidate variables parity, day of lactation, dietary nutrient composition, milk yield, milk composition, and the milk FA profile were provided which resulted in a pool of 62 potential predictors. The prediction of EB was performed in two different ways: first, an automated stepwise variable selection was performed with the whole variable pool (GLMs-N) and with FA only (GLMs-FA-N). As this method recently earned criticism, some other methods were also tested for a first variable selection: the regularized linear regression models Lasso, elastic net (ENET), adaptive Lasso (AdaLasso), and adaptive elastic net (ADAENET). As a machine learning method which also considers interactions and non-linear relationships random forests were also applied. The first variable selection was performed using a five-fold cross-validation which resulted in five models per selection method. All chosen effects were combined to one model (MODEL1) for each method, respectively. For random forests the 30 most important effects were chosen. Following this, the individual effects of the MODEL1 were used for a forward selection based on the corrected Akaike Information Criterion (AICC) for further model reduction, resulting in MODEL2. Then, the non-significant effects were removed from the MODEL2, achieving the final MODEL3 for each method. As MODEL2 of AdaLasso did show no improvement compared with MODEL1 this step was skipped and non-significant effects

were directly removed from MODEL1, resulting in MODEL4. The final models were validated using leave-one-out cross-validation.

The models showed adequate correlations (r) between the predicted and the observed EB in leave-one-out cross-validation: although GLMs-FA-N had the lowest accuracy (r = 0.79), the result was still remarkable and showed how much information milk FA alone can provide. GLMs-N and AdaLasso performed best with r = 0.86 and 0.85 containing 21 and 18 predictors, respectively. However, other models like ADAENET achieved only slightly lower accuracy (r = 0.83) with only 6 predictors. The composition of the predictors was relatively similar in all models. All (except for GLMs-FA-N) contained days in milk, milk yield, C18:1c9, C15:0*iso,* and the ratio of *omega-6* to *omega-3* FA (*n-6*/*n-3*) as effects with the strongest impacts on the prediction. While milk yield, days in milk, and C18:1c9 mirrored physiologically obvious effects, the strong and positive impact of *n-6*/*n-3* and C15:0*iso* was unexpected. The n-*6*/*n-3* ratio might be physiologically connected to EB as it reflects the dietary forage-to-concentrate ratio in some way: being the main *n-3* FA, α-linoleic acid supply increases with increasing grass-based forages in the diet, while the *n-6* FA supply (mainly linoleic acid) is supposed to increase with increasing grain (energy) content. It is likely that this effect was reflected in the models because it is largely independent from other FA, which are often highly intercorrelated because of their common origin. The importance of C15:0*iso*, a FA arising from microbial FA synthesis in the rumen, could not be explained satisfyingly. The nature of the potential physiological connections between EB and some FA like C15:0*iso* or *n-6* or *n-3* FA might require further research. There were no effects of dietary composition in the models, except for RNB (ruminal-N-balance) in the AdaLasso and the GLMs-N derived models. This might be a consequence of the low variation which existed in the nutrient composition of the diets that were used in the research stations.

The present study showed that it is possible to predict the cow's EB from animal and milk traits with an adequate accuracy. As long as the diets have similar composition and not contain ingredients which strongly affect the milk FA profile, dietary effects have not to be taken into account. However, a practical application of the obtained models is not yet possible: First, as the dataset was relatively small (n = 248), it is not clear whether or not the models would perform adequately with independent datasets. Second, FA analysis by gas chromatography is very expensive. Third, even if gas chromatographic analysis were affordable for standard milk analysis, there are some highly variable, very low concentrated FA as predictors in the models, which might be prone to laboratory effects, and this could spoil the predictions.

Although under criticism, automatic stepwise selection provided the best performing model and thus seems sufficient for practical issues like the one dealt with in the present study. However, the differences in accuracy between the applied methods were very small and as regularized

linear regression methods, especially ENET and ADAENET, are supposed to deal better with highly correlated variables, it might be safer to use them with datasets containing highly correlated variables such as the one used in the present work.

6 ZUSAMMENFASSUNG

In der Frühlaktation, wenn die Futteraufnahme nicht den Energiebedarf, der durch die hohe Milchleistung entsteht, decken kann, geraten Milchkühe in eine negative Energiebilanz (NEB). Die Tiere reagieren darauf zum Ausgleich mit der Mobilisierung von Körperreserven, was grundsätzlich ein natürlicher Prozess ist. Allerdings verstärkte die jahrzehntelange Züchtung auf hohe Milchleistung die Diskrepanz zwischen Energieaufnahme und –bedarf so stark, dass dadurch bedingt Stoffwechselkrankheiten wie Ketose und Fettleber oder eine eingeschränkte Fruchtbarkeit ebenfalls stark zunahmen. Das Monitoring der Energiebilanz (EB) gewann in den letzten Jahren zunehmend an Bedeutung: einerseits gedacht als Warnung für das Management, wenn Tiere in eine sehr tiefe EB für zu lange Zeit geraten, andererseits als mögliches Tool zur Selektion von Tieren, die mit der Stoffwechselbelastung besonders gut umgehen können. Allerdings ist dies schwierig, da in der Regel die individuelle Futteraufnahme nicht gemessen werden kann und darum auf Indikatoren und Schätzungen zurückgegriffen werden muss.

Ziel dieser Doktorarbeit war es, die EB anhand von Tierdaten, Futterinformationen und der Milchzusammensetzung zu schätzen. Da das Muster der Milchfettsäuren bekanntermaßen auf physiologische Gegebenheiten wie Energiemangel reagiert, wurde hierauf besonderes Augenmerk gelegt um neue Fettsäuren zu identifizieren, die als Indikator für NEB dienen könnten. Dazu wurden von sechs Versuchsstationen in Deutschland einzelne Milchproben von Kühen zwischen dem 6. und 133. Laktationstag gezogen um eine möglichst breites Spektrum an Rationen und EB abzudecken. Die Milchzusammensetzung wurde mit Hilfe von Infrarotspektroskopie analysiert, die Milchfettzusammensetzung gaschromatographisch (GC). Die EB (MJ NEL/d) als Response-Variable wurde als die Differenz des kalkulierten Energiebedarfs von der Energieaufnahme berechnet. Als potenzielle Prädiktorvariablen kamen Laktationsnummer, -tag, die Nährstoffzusammensetzung der Ration, Milchleistung, Milchinhaltsstoffe und das Milchfettsäuremuster infrage – insgesamt ein Pool von 62 Variablen. Für die Schätzung wurden zwei verschiedene Strategien angewandt: zuerst eine automatisierte, schrittweise Variablenselektion, die einmal mit dem gesamten Variablenpool durchgeführt wurde (GLMs-N) und einmal allein mit den Milchfettsäuren (GLMs-FA-N). Da diese Methode jedoch in der Kritik steht, wurden zum Vergleich für eine erste Variablenselektion andere Methoden angewandt: die regularisierten linearen Regressionsmethoden „Lasso“, „Elastic net“ (ENET), „adaptive Lasso“ (AdaLasso) und „adaptive elastic net“ (ADAENET). Als Referenz, die auch Interaktionen und nicht-lineare Beziehungen berücksichtigt, wurde die „Machine-Learning“-Methode „Random Forests“ eingesetzt. Zunächst wurden zur Variablenselektion diese Methoden auf den ganzen Variablenpool mit einer fünffachen Kreuzvalidierung angewandt, was zu fünf Modellen pro

Methode führte. Die ausgewählten Variablen dieser fünf Modelle wurden jeweils zu einem Modell (MODEL1) zusammengefasst. Bei „Random Forests“ wurden hierfür die 30 laut Programm wichtigsten Variablen genutzt. Anschließend wurde mit den Variablen der MODEL1 eine Vorwärtsselektion anhand des korrigierten „Akaike Information Criterion“ (AICC) durchgeführt, welche zu den weiter reduzierten MODEL2 führte. In einem dritten Schritt entstanden die MODEL3 durch das sukzessive Entfernen der nicht-signifikanten Effekte. Bei AdaLasso führte der zweite Schritt zu keiner Verbesserung des Modells, daher wurden die nicht-signifikanten Effekte gleich aus MODEL1 entfernt (MODEL4). Die letzten Modelle wurden mit Hilfe einer „Leave-one-out“-Kreuzvalidierung validiert.

Die Modelle zeigten in der „Leave-one-out“-Kreuzvalidierung recht gute Korrelationen zwischen der berechneten und geschätzten EB: die Genauigkeit von GLMs-N und AdaLasso war mit r = 0.86 und 0.85 die höchste. Die Modelle enthielten jeweils 21 und 18 Effekte. Allerdings konnten andere Modelle, wie z.B. ADAENET mit nur 6 Effekten eine ähnlich hohe Genauigkeit erzielen (r = 0.83). Obwohl GLMs-FA-N die geringste Schätzgenauigkeit aufwies (r = 0.79), ist es doch erstaunlich wie viel Information die Milchfettsäuren allein liefern können. Die Zusammensetzung der Effekte war bei den Modellen recht ähnlich: abgesehen von GLMs-FA-N enthielten alle Modelle als stärkste Effekte Laktationstag, Milchleistung, C18:1c9, 15:0*iso* und das Verhältnis zwischen Omega-6- und Omega-3-Fettsäuren (*n-6*/*n-3*). Während Laktationstag, Milchleistung und C18:1c9 physiologisch gesehen einen eindeutigen Zusammenhang zur EB haben, war dieser bei 15:0*iso* und *n-6*/*n-3* zunächst nicht klar. *N-6*/*n-3* könnte das Grundfutter-Kraftfutter-Verhältnis widerspiegeln, welches Einfluss auf die Energiedichte und damit die EB haben kann: als Hauptbestandteil der *n-3*-Fettsäuren könnte α-Linolensäure in der Milch ansteigen, wenn der Anteil an (grasbasiertem) Grundfutter steigt (und damit die Energiedichte sinkt), während die Linolsäure als hauptsächlich vorkommende *n-6*-Fettsäure eher in Getreide vertreten ist und damit für eine steigende Energiedichte stünde. Es ist wahrscheinlich, dass dieser Effekt auch seinen Weg in die Modelle fand, weil er weitestgehend unabhängig von den anderen Milchfettsäuren ist, die untereinander teilweise stark korreliert sind. Die Bedeutung von C15:0*iso* als Fettsäure, die hauptsächlich von der mikrobiellen Fettsäuresynthese im Pansen herrührt, konnte nicht vollständig erklärt werden. Um den Zusammenhang zwischen diesen und evtl. einigen anderen Fettsäuren und der EB zu erklären wären weitere Untersuchungen nötig. Abgesehen von der RNB (ruminale N-Bilanz) in den Modellen, die aus AdaLasso und GLMs-N entstanden, erschienen keine Rationseffekte. Dies steht vermutlich mit der geringen Varianz der Nährstoffgehalte in den verschiedenen Rationen in Zusammenhang.

Die vorliegende Arbeit zeigte, dass es möglich ist, die EB mit Hilfe von Tierdaten, Milchinhaltsstoffen und dem Milchfettsäuremuster mit relativ guter Genauigkeit zu schätzen.

Nährstoffgehalte der Rationen scheinen vernachlässigbar, zumindest solange keine Rationskomponenten eingesetzt werden, die sich auf besondere Weise auf das Milchfettsäuremuster auswirken. Dennoch ist eine praktische Anwendung dieser Modelle derzeit unwahrscheinlich: zunächst ist der Datensatz mit nur 248 Beobachtungen relativ klein, was es unwahrscheinlich macht, dass die Modelle stabil genug sind um angewandt auf unabhängige Datensätze ähnlich gute Ergebnisse erzielen würden. Zweitens ist die Fettsäureanalyse mittels GC bei Weitem zu teuer für praktische Betriebe. Drittens, selbst wenn dies nicht der Fall wäre, die Modelle enthalten einige sehr gering konzentrierte, hochvariable Fettsäuren, die vermutlich sehr anfällig für Laboreffekte sind und auch dadurch die Genauigkeit der Schätzungen im Einsatz beeinträchtigen könnten.

Trotz der bestehenden Kritik an der automatisierten Variablenselektion zeigte das darauf basierende Modell durchweg die beste Leistung und scheint daher vom praktischen Aspekt her für Zwecke wie die der vorliegenden Studie dennoch geeignet. Allerdings waren die Unterschiede in der Schätzgenauigkeit zwischen den Methoden sehr gering und da die regularisierten Regressionsmethoden, besonders ENET und ADAENET, grundsätzlich besser mit hoch korrelierten Variablen umgehen können, sollten sie bei Datensätzen wie dem der vorliegenden Studie bevorzugt werden.

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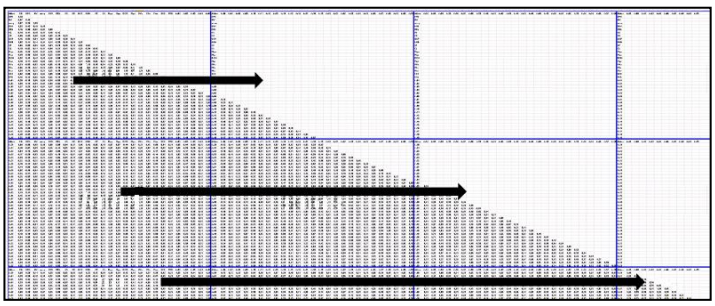
8 ANNEX

Annex 1 Abbreviations (Abbr) used for the correlation matrix in Annex 3 in the order of occurrence (FA in g/100 g FA)

Variable	Abbrev	Variable	Abbrev
Energy balance (MJ NEL/d)	EB	C18:0	fa24
Dry matter intake (kg/d)	DMI	C18:0*iso*	fa25
Body weight (kg)	BW	C18:1t6-t11	fa26
Requirement for maintenance (MJ NEL/d)	mreq	C18:1t12-t14	fa27
Days in milk (d)	DIM	C18:1c9	fa28
Net energy for lactation (MJ/kg DM)	NEL	C18:1c11	fa29
Organic matter (g/kg DM)	OS	C18:1c12	fa30
Crude protein (g/kg DM)	CP	C18:1c13+c14+t16	fa31
Undegraded protein at the duodenum (g/kg DM)	UCP	C18:2t,t-NMID	fa32
Ruminal nitrogen balance (g/kg DM)	RNB	C18:2c9,t11	fa33
Crude fiber (g/kg DM)	CF	C18:2c9,t13+t8,c12	fa34
Crude fat (g/kg DM)	CL	C18:2c9,t12+t8,c13	fa35
Milk yield, week's mean (kg/d)	MYw	C18:2t11,c15+t9,c12	fa36
Milk yield at sampling (kg/d)	MYs	C18:2c9,c12	fa37
Energy-corrected milk yield (kg/d)	ECM	C18:3c9,c12,c15	fa38
Milk Protein (%)	Mpr	C19:0	fa39
Milk Fat (%)	Mfa	C20:0	fa40
Milk Lactose (%)	Mla	C20:1c9	fa41
Urea (mg/dl)	Mur	C20:1c11	fa42
Somatic cell score (k)	SCC	C20:3*n-6*	fa43
Fat/Protein	FPR	C20:4*n-6*	fa44
C4:0	fa01	C20:5*n-3*	fa45
C6:0	fa02	C22:0	fa46
C8:0	fa03	∑C18:1*cis*	fa48
C10:0	fa04	∑C18:1*trans*	fa49
C10:1	fa05	∑C18:2	fa50
C12:0	fa06	∑*trans*	fa51
C12:1*cis*+C13:0	fa07	BCFA, branched chain FA	fa52
C13:0*iso*	fa08	∑*n-3*	fa53
C13:0*anteiso*	fa09	∑*n-6*	fa54
C14:0	fa10	SFA, saturated FA	fa55
C14:1*cis*	fa11	MUFA, monounsaturated FA	fa56
C14:0*iso*	fa12	PUFA, polyunsaturated FA	fa57
C15:0	fa13	UFA, unsaturated FA	fa58
C15:0*iso*	fa14	SCFA, short-chain FA	fa59
C15:0*anteiso*	fa15	MCFA, medium-chain FA	fa60
C16:0	fa16	LCFA, long-chain-FA	fa61
C16:1*trans*	fa17	*n-6/n-3*	fa63
C16:1*cis*	fa18	∑C4-15	fa65
C16:0*iso*	fa19	∑C16	fa66
C17:0	fa20	∑C6-15	fa67
C17:1	fa21	C6-15/LCFA	fa68
C17:0*iso*	fa22	OCFA, odd-chain FA	fa69
C17:0*anteiso*	fa23	C15/C17	fa78

c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA, *n-6* = o*mega-6* FA

Annex 2 Scheme of the correlation matrix displayed in Annex 3



Annex 3 (1/9) Pearson correlations between all variables of the dataset (see Annex 1 for the abbreviation code (Abbr)).

Abbr	EB	DMI	BW	mreq	DIM	NEL	OS	CP	UCP	RNB	CF	CL	MYw	MYs	ECM	Mpr	Mfa	Mla	Mur	SCC	FPR	fa01	fa02	fa03
DMI	0.50																							
BW	n.s.	0.46																						
mreq	n.s.	0.46	1.00																					
DIM	0.55	0.42	n.s.	n.s.																				
NEL	0.16	n.s.	n.s.	n.s.	n.s.																			
OS	-0.14	0.17	n.s.	n.s.	n.s.	n.s.																		
CP	n.s.	n.s.	n.s.	n.s.	n.s.	-0.13	0.43																	
UCP	-0.16	n.s.	n.s.	n.s.	n.s.	0.28	0.50	0.81																
RNB	n.s.	n.s.	0.14	0.14	n.s.	-0.58	n.s.	0.68	n.s.															
CF	n.s.	n.s.	n.s.	n.s.	n.s.	-0.70	-0.30	0.13	-0.32	0.62														
CL	-0.19	n.s.	n.s.	n.s.	n.s.	0.21	0.45	n.s.	0.29	-0.34	-0.43													
MYw	-0.46	0.44	0.40	0.41	-0.16	n.s.	0.35	n.s.	0.18	n.s.	-0.21	0.22												
MYs	-0.49	0.38	0.38	0.39	-0.22	n.s.	0.35	n.s.	0.19	n.s.	-0.18	0.22	0.95											
ECM	-0.52	0.45	0.42	0.43	-0.16	n.s.	0.30	n.s.	0.17	n.s.	-0.15	0.21	0.92	0.89										
Mpr	0.40	n.s.	n.s.	n.s.	0.35	0.17	-0.15	n.s.	n.s.	n.s.	n.s.	-0.22	-0.42	-0.40	-0.28									
Mfa	-0.21	n.s.	n.s.	n.s.	n.s.	n.s.	-0.20	n.s.	n.s.	0.13	0.18	n.s.	-0.26	-0.20	n.s.	0.26								
Mla	n.s.	-0.18	-0.27	-0.26	n.s.	n.s.	n.s.	n.s.	n.s.	-0.21	-0.14	0.23	n.s.	n.s.	n.s.	-0.14	n.s.							
Mur	0.31	0.14	-0.22	-0.21	0.25	-0.18	0.20	0.30	0.17	0.29	n.s.	n.s.	n.s.	-0.15	-0.19	0.17	-0.24	n.s.						
SCC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.16	n.s.	-0.13	n.s.	0.13	n.s.	n.s.	n.s.	n.s.	-0.26	n.s.					
FPR	-0.42	-0.15	n.s.	n.s.	-0.25	-0.19	-0.13	n.s.	-0.14	n.s.	0.17	n.s.	n.s.	n.s.	0.26	-0.26	0.86	n.s.	-0.32	n.s.				
fa01	-0.30	-0.18	n.s.	n.s.	-0.34	n.s.	n.s.	-0.13	-0.14	n.s.	n.s.	n.s.	n.s.	0.19	0.13	-0.27	0.13	n.s.	-0.23	n.s.	0.27			
fa02	n.s.	0.14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.13	n.s.	n.s.	n.s.	0.26		
fa03	0.27	0.26	n.s.	n.s.	0.17	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.19	n.s.	n.s.	0.15	n.s.	n.s.	n.s.	0.91	
fa04	0.41	0.36	n.s.	n.s.	0.35	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.14	n.s.	0.36	n.s.	n.s.	0.21	n.s.	-0.15	-0.18	0.71	0.92
fa05	0.35	0.31	n.s.	n.s.	0.31	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.23	n.s.	n.s.	0.18	n.s.	-0.19	-0.17	0.51	0.67
fa06	0.55	0.46	n.s.	n.s.	0.51	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.17	-0.13	-0.19	n.s.	0.47	n.s.	n.s.	0.31	n.s.	-0.26	-0.32	0.51	0.78
fa07	0.54	0.41	n.s.	n.s.	0.47	0.18	n.s.	n.s.	n.s.	n.s.	-0.15	n.s.	n.s.	-0.14	-0.13	0.47	-0.16	-0.17	0.19	n.s.	-0.41	-0.40	0.14	0.43
fa08	0.33	0.20	n.s.	n.s.	0.24	n.s.	n.s.	-0.13	n.s.	n.s.	-0.13	n.s.	n.s.	n.s.	n.s.	0.15	-0.13	n.s.	0.26	n.s.	-0.21	n.s.	n.s.	n.s.
fa09	0.47	0.39	n.s.	n.s.	0.52	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.14	n.s.	0.35	n.s.	-0.16	0.38	n.s.	-0.31	-0.35	0.26	0.48
fa10	0.62	0.51	n.s.	n.s.	0.57	n.s.	n.s.	n.s.	n.s.	0.15	n.s.	-0.25	n.s.	-0.14	-0.14	0.38	-0.18	n.s.	0.36	n.s.	-0.39	-0.32	0.35	0.60
fa11	0.38	0.37	n.s.	n.s.	0.51	n.s.	n.s.	n.s.	n.s.	0.16	n.s.	-0.14	n.s.	n.s.	n.s.	0.27	-0.19	-0.21	0.32	n.s.	-0.33	-0.38	n.s.	n.s.
fa12	0.28	n.s.	-0.19	-0.20	n.s.	n.s.	n.s.	n.s.	n.s.	0.15	n.s.	-0.24	-0.23	-0.23	-0.26	n.s.	n.s.	n.s.	0.31	n.s.	n.s.	n.s.	0.27	0.24
fa13	0.51	0.32	n.s.	n.s.	0.41	0.17	n.s.	n.s.	n.s.	n.s.	n.s.	-0.13	-0.13	-0.16	-0.20	0.41	-0.24	-0.15	0.14	n.s.	-0.45	-0.34	n.s.	0.24
fa14	0.45	0.18	-0.15	-0.15	0.30	n.s.	n.s.	n.s.	n.s.	0.13	0.14	n.s.	-0.23	-0.26	-0.30	0.19	-0.20	0.15	0.42	n.s.	-0.30	-0.18	0.26	0.33
fa15	0.46	0.21	0.13	0.13	0.40	n.s.	n.s.	0.24	0.17	0.20	0.15	-0.31	-0.16	-0.19	-0.28	0.25	-0.36	n.s.	0.24	n.s.	-0.51	-0.21	n.s.	0.26

Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant.

Annex 3 continued (2/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	fa04	fa05	fa06	fa07	fa08	fa09	fa10	fa11	fa12	fa13	fa14	fa15	fa16	fa17	fa18	fa19	fa20	fa21	fa22	fa23	fa24	fa25	fa26	fa27
DMI																								
BW																								
mreq																								
DIM																								
NEL																								
OS																								
CP																								
UCP																								
RNB																								
CF																								
CL																								
MYw																								
MYs																								
ECM																								
Mpr																								
Mfa																								
Mla																								
Mur																								
SCC																								
FPR																								
fa01																								
fa02																								
fa03																								
fa04																								
fa05	0.68																							
fa06	0.93	0.65																						
fa07	0.63	0.66	0.76																					
fa08	n.s.	n.s.	0.25	n.s.																				
fa09	0.58	0.78	0.71	0.68	0.24																			
fa10	0.77	0.54	0.90	0.70	0.39	0.65																		
fa11	0.23	0.52	0.42	0.51	0.13	0.80	0.49																	
fa12	0.20	n.s.	0.19	n.s.	0.48	n.s.	0.26	n.s.																
fa13	0.45	0.45	0.57	0.89	n.s.	0.44	0.60	0.35	n.s.															
fa14	0.37	0.15	0.40	0.14	0.57	0.26	0.51	n.s.	0.67	0.17														
fa15	0.39	0.21	0.46	0.41	0.23	0.27	0.59	0.23	0.28	0.56	0.59													

Annex 3 continued (3/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	EB	DMI	BW	mreq	DIM	NEL	OS	CP	UCP	RNB	CF	CL	MYw	MYs	ECM	Mpr	Mfa	Mla	Mur	SCC	FPR	fa01	fa02	fa03
fa16	0.46	0.38	n.s.	n.s.	0.48	-0.13	n.s.	n.s.	-0.22	0.26	0.20	-0.41	-0.20	-0.21	-0.13	0.33	0.14	-0.32	0.17	n.s.	n.s.	-0.23	n.s.	0.20
fa17	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.28	0.18	0.22	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.13	n.s.	n.s.
fa18	-0.28	n.s.	0.23	0.24	-0.18	-0.19	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.13	0.20	n.s.	0.17	-0.32	-0.19	n.s.	0.23	n.s.	-0.47	-0.54
fa19	0.25	n.s.	-0.20	-0.20	n.s.	n.s.	n.s.	n.s.	n.s.	0.16	0.18	-0.15	-0.22	-0.22	-0.23	0.14	n.s.	n.s.	0.21	n.s.	n.s.	n.s.	0.18	0.16
fa20	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.17	n.s.	0.16	n.s.	-0.13	n.s.	n.s.	-0.14	n.s.	-0.21	-0.15	n.s.	n.s.	-0.24	n.s.	-0.41	-0.39
fa21	n.s.	n.s.	n.s.	n.s.	-0.16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.20	-0.20
fa22	n.s.	-0.16	n.s.	n.s.	n.s.	n.s.	n.s.	0.14	0.17	n.s.	n.s.	n.s.	n.s.	n.s.	-0.15	-0.19	-0.39	0.13	0.17	n.s.	-0.29	-0.15	-0.17	-0.19
fa23	-0.17	-0.20	n.s.	n.s.	-0.29	n.s.	-0.14	n.s.	n.s.	n.s.	0.15	n.s.	n.s.	n.s.	n.s.	-0.16	n.s.	n.s.	-0.14	n.s.	0.13	n.s.	0.36	0.30
fa24	-0.33	-0.39	-0.25	-0.25	-0.42	n.s.	n.s.	-0.19	n.s.	-0.26	n.s.	0.32	n.s.	n.s.	n.s.	-0.25	0.17	0.37	-0.16	n.s.	0.30	0.35	n.s.	-0.14
fa25	-0.55	-0.56	-0.25	-0.24	-0.60	n.s.	n.s.	0.16	0.19	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.32	n.s.	n.s.	-0.13	n.s.	0.25	0.19	-0.13	-0.32
fa26	-0.13	n.s.	n.s.	n.s.	n.s.	0.24	0.31	0.17	0.36	-0.16	-0.35	0.45	0.21	0.16	n.s.	-0.14	-0.36	n.s.	n.s.	n.s.	-0.30	-0.13	-0.27	-0.26
fa27	n.s.	n.s.	n.s.	n.s.	n.s.	0.19	0.36	0.27	0.37	n.s.	-0.28	0.40	0.24	0.22	n.s.	n.s.	-0.32	n.s.	n.s.	n.s.	-0.26	n.s.	-0.13	n.s.
fa28	-0.62	-0.46	n.s.	n.s.	-0.59	n.s.	n.s.	n.s.	n.s.	-0.17	n.s.	0.22	0.18	0.22	0.18	-0.42	n.s.	0.14	-0.28	n.s.	0.26	0.22	-0.41	-0.61
fa29	-0.55	-0.41	n.s.	n.s.	-0.64	n.s.	n.s.	n.s.	0.16	n.s.	n.s.	0.16	0.21	0.23	0.17	-0.34	n.s.	n.s.	-0.34	n.s.	0.13	0.14	-0.35	-0.48
fa30	-0.26	n.s.	n.s.	n.s.	n.s.	n.s.	0.29	n.s.	n.s.	n.s.	-0.31	0.37	0.25	0.24	0.19	-0.27	n.s.	n.s.	-0.22	n.s.	n.s.	0.20	n.s.	n.s.
fa31	-0.40	-0.29	n.s.	n.s.	-0.33	0.16	n.s.	n.s.	0.19	n.s.	-0.25	0.30	0.19	0.20	0.15	-0.28	n.s.	n.s.	-0.31	n.s.	n.s.	0.26	-0.28	-0.35
fa32	0.22	n.s.	n.s.	n.s.	0.37	n.s.	0.20	n.s.	n.s.	0.14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.23	n.s.	0.23	n.s.	-0.29	-0.20	n.s.	n.s.
fa33	n.s.	n.s.	n.s.	n.s.	0.18	0.27	0.37	0.27	0.39	n.s.	-0.40	0.27	n.s.	n.s.	n.s.	n.s.	-0.35	n.s.	n.s.	n.s.	-0.34	-0.13	-0.29	-0.22
fa34	n.s.	n.s.	0.21	0.21	0.27	0.15	0.30	0.23	0.29	n.s.	-0.23	0.20	0.18	0.15	n.s.	n.s.	-0.41	n.s.	n.s.	n.s.	-0.41	-0.13	-0.34	-0.23
fa35	n.s.	n.s.	0.20	0.20	0.31	n.s.	0.22	0.24	0.23	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.31	n.s.	n.s.	n.s.	-0.34	n.s.	-0.42	-0.35
fa36	-0.15	n.s.	n.s.	n.s.	-0.13	-0.16	0.15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.17	n.s.	n.s.	0.16	0.13
fa37	n.s.	n.s.	0.19	0.19	n.s.	0.37	n.s.	n.s.	0.23	-0.22	-0.30	0.18	0.18	0.15	n.s.	n.s.	-0.26	n.s.	-0.17	n.s.	-0.21	n.s.	-0.20	-0.15
fa38	-0.28	-0.18	n.s.	n.s.	n.s.	-0.14	0.28	0.46	0.41	0.27	n.s.	n.s.	0.15	0.15	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
fa39	n.s.	n.s.	n.s.	n.s.	0.16	n.s.	0.15	n.s.	0.13	n.s.	-0.14	0.14	n.s.	n.s.	n.s.	n.s.	-0.30	n.s.	n.s.	n.s.	-0.28	n.s.	-0.57	-0.52
fa40	n.s.	-0.17	-0.26	-0.26	n.s.	0.17	-0.18	-0.25	n.s.	-0.29	n.s.	0.34	-0.18	-0.15	-0.15	n.s.	n.s.	0.32	n.s.	n.s.	0.15	0.14	0.19	n.s.
fa41	0.27	n.s.	n.s.	n.s.	0.33	0.13	n.s.	-0.27	-0.17	-0.24	n.s.	0.25	n.s.	n.s.	-0.14	n.s.	-0.32	0.19	n.s.	n.s.	-0.29	-0.17	n.s.	n.s.
fa42	-0.38	-0.35	n.s.	n.s.	-0.32	0.26	n.s.	n.s.	0.23	-0.25	-0.24	0.43	0.15	0.14	n.s.	-0.25	n.s.	0.20	n.s.	n.s.	n.s.	n.s.	-0.33	-0.39
fa43	0.39	0.28	0.25	0.25	0.43	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.14	-0.16	0.23	-0.14	n.s.	n.s.	n.s.	-0.27	-0.14	n.s.	0.26
fa44	n.s.	-0.18	0.16	0.16	n.s.	0.20	-0.14	n.s.	n.s.	n.s.	n.s.	n.s.	-0.21	-0.21	-0.28	n.s.	-0.14	n.s.	n.s.	n.s.	-0.17	0.13	n.s.	n.s.
fa45	-0.23	-0.19	n.s.	n.s.	-0.16	-0.17	n.s.	-0.17	-0.23	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.25	n.s.	n.s.	n.s.	n.s.	n.s.	0.26	n.s.	n.s.
fa46	n.s.	-0.24	-0.22	-0.22	-0.16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.17	-0.16	-0.14	n.s.	0.13	n.s.	n.s.	n.s.	0.15	n.s.	n.s.	n.s.
fa48	-0.63	-0.47	n.s.	n.s.	-0.60	n.s.	n.s.	n.s.	n.s.	-0.17	n.s.	0.22	0.19	0.23	0.18	-0.43	n.s.	0.13	-0.29	n.s.	0.25	0.22	-0.41	-0.61
fa49	-0.13	n.s.	n.s.	n.s.	n.s.	0.24	0.35	0.22	0.39	n.s.	-0.36	0.47	0.24	0.20	n.s.	-0.14	-0.38	n.s.	n.s.	n.s.	-0.31	n.s.	-0.25	-0.22
fa50	n.s.	n.s.	0.18	0.18	0.15	0.32	0.24	0.17	0.32	n.s.	-0.35	0.24	0.19	0.17	n.s.	n.s.	-0.39	n.s.	n.s.	n.s.	-0.35	n.s.	-0.30	-0.23
fa51	n.s.	n.s.	n.s.	n.s.	n.s.	0.22	0.36	0.23	0.38	n.s.	-0.34	0.44	0.23	0.19	n.s.	n.s.	-0.40	n.s.	n.s.	n.s.	-0.34	n.s.	-0.26	-0.23

Annex 3 continued (4/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	fa04	fa05	fa06	fa07	fa08	fa09	fa10	fa11	fa12	fa13	fa14	fa15	fa16	fa17	fa18	fa19	fa20	fa21	fa22	fa23	fa24	fa25	fa26	fa27
fa16	0.32	0.45	0.47	0.51	n.s.	0.51	0.54	0.49	n.s.	0.51	n.s.	0.18												
fa17	n.s.	0.24	n.s.	n.s.	-0.16	n.s.	-0.13	n.s.	n.s.	-0.14	n.s.	n.s.	n.s.											
fa18	-0.51	-0.29	-0.44	-0.17	-0.32	-0.15	-0.45	0.15	-0.44	-0.13	-0.55	-0.40	n.s.	n.s.										
fa19	0.16	n.s.	0.17	-0.14	0.46	n.s.	0.25	n.s.	0.73	n.s.	0.60	0.28	n.s.	n.s.	-0.37									
fa20	-0.25	-0.42	n.s.	0.14	n.s.	-0.17	n.s.	n.s.	-0.14	0.37	n.s.	0.32	n.s.	-0.35	0.23	n.s.								
fa21	-0.17	n.s.	-0.15	n.s.	n.s.	n.s.	-0.13	n.s.	-0.13	n.s.	n.s.	n.s.	n.s.	n.s.	0.20	-0.13	0.13							
fa22	-0.19	-0.31	-0.18	-0.22	n.s.	-0.16	n.s.	n.s.	0.17	n.s.	0.45	0.44	-0.47	n.s.	-0.15	0.22	0.35	n.s.						
fa23	0.16	0.35	n.s.	n.s.	-0.44	n.s.	-0.22	-0.24	n.s.	n.s.	n.s.	n.s.	-0.22	0.41	-0.20	-0.14	-0.44	n.s.	n.s.					
fa24	-0.30	-0.48	-0.44	-0.61	0.14	-0.61	-0.49	-0.69	0.16	-0.57	n.s.	-0.31	-0.65	n.s.	-0.29	0.22	-0.16	n.s.	0.14	n.s.				
fa25	-0.46	-0.40	-0.61	-0.58	-0.30	-0.55	-0.68	-0.50	n.s.	-0.46	-0.16	-0.23	-0.58	0.14	0.16	n.s.	n.s.	0.13	0.30	0.41	0.40			
fa26	-0.25	-0.19	-0.23	n.s.	n.s.	n.s.	-0.22	n.s.	n.s.	n.s.	n.s.	n.s.	-0.45	0.23	n.s.	n.s.	n.s.	n.s.	0.50	n.s.	n.s.	0.18		
fa27	n.s.	n.s.	n.s.	n.s.	-0.18	n.s.	n.s.	n.s.	-0.24	n.s.	-0.14	n.s.	-0.33	0.26	-0.16	-0.25	n.s.	n.s.	0.26	n.s.	n.s.	n.s.	0.67	
fa28	-0.74	-0.61	-0.85	-0.72	-0.30	-0.65	-0.86	-0.45	-0.22	-0.65	-0.36	-0.47	-0.74	n.s.	0.40	-0.18	n.s.	0.16	0.26	0.18	0.46	0.72	0.20	n.s.
fa29	-0.57	-0.43	-0.67	-0.44	-0.42	-0.57	-0.71	-0.40	-0.34	-0.33	-0.44	-0.35	-0.64	n.s.	0.38	-0.37	n.s.	0.24	0.19	0.30	0.28	0.66	0.34	0.19
fa30	-0.17	n.s.	-0.25	n.s.	-0.26	n.s.	-0.32	n.s.	-0.24	-0.19	-0.35	-0.23	-0.33	0.48	n.s.	-0.32	-0.24	n.s.	n.s.	0.20	n.s.	n.s.	0.42	0.71
fa31	-0.42	-0.36	-0.50	-0.33	-0.30	-0.43	-0.54	-0.32	-0.33	-0.29	-0.40	-0.23	-0.62	0.19	n.s.	-0.33	n.s.	0.17	0.15	n.s.	0.35	0.32	0.46	0.64
fa32	n.s.	0.20	0.21	0.24	n.s.	0.33	0.22	0.40	0.16	0.19	0.17	0.18	n.s.	0.20	-0.14	n.s.	n.s.	n.s.	0.23	n.s.	-0.27	-0.20	0.54	0.45
fa33	-0.16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.24	n.s.	n.s.	n.s.	0.13	-0.17	0.33	n.s.	n.s.	n.s.	n.s.	0.33	-0.13	-0.27	n.s.	0.72	0.55
fa34	n.s.	n.s.	n.s.	0.27	-0.24	0.15	n.s.	0.32	-0.35	0.32	-0.19	0.24	n.s.	0.15	0.20	-0.34	0.33	n.s.	0.27	-0.16	-0.41	-0.15	0.57	0.76
fa35	-0.19	-0.38	n.s.	n.s.	0.14	n.s.	0.15	0.25	-0.17	0.20	n.s.	0.29	n.s.	-0.23	0.17	n.s.	0.65	n.s.	0.33	-0.70	-0.21	-0.26	0.33	0.47
fa36	n.s.	0.35	n.s.	n.s.	-0.23	n.s.	-0.19	n.s.	0.13	n.s.	n.s.	-0.13	-0.19	0.50	-0.15	n.s.	-0.45	n.s.	n.s.	0.58	n.s.	0.23	0.39	0.31
fa37	-0.15	-0.16	-0.13	n.s.	-0.22	-0.13	-0.15	n.s.	-0.25	n.s.	-0.30	n.s.	-0.27	n.s.	n.s.	-0.17	0.16	n.s.	0.21	n.s.	n.s.	n.s.	0.35	0.38
fa38	n.s.	-0.15	-0.14	-0.18	-0.16	n.s.	-0.16	n.s.	n.s.	n.s.	n.s.	0.16	-0.30	0.26	n.s.	n.s.	0.15	n.s.	0.40	n.s.	n.s.	0.27	0.42	0.54
fa39	-0.40	-0.43	-0.23	n.s.	0.13	-0.16	n.s.	0.17	-0.13	n.s.	n.s.	n.s.	-0.15	-0.20	0.17	n.s.	0.52	n.s.	0.35	-0.62	n.s.	n.s.	0.51	0.50
fa40	n.s.	-0.18	n.s.	-0.25	0.16	-0.29	n.s.	-0.41	0.22	-0.23	0.29	n.s.	-0.37	n.s.	-0.46	0.28	-0.15	n.s.	0.18	n.s.	0.70	n.s.	n.s.	n.s.
fa41	n.s.	0.24	n.s.	0.13	0.20	0.23	0.19	0.23	0.13	n.s.	0.37	0.30	-0.15	n.s.	-0.36	n.s.	n.s.	-0.19	0.35	n.s.	n.s.	-0.21	0.14	n.s.
fa42	-0.43	-0.42	-0.48	-0.34	n.s.	-0.40	-0.47	-0.25	-0.14	-0.30	-0.15	-0.16	-0.68	n.s.	n.s.	n.s.	n.s.	n.s.	0.46	n.s.	0.38	0.46	0.61	0.28
fa43	0.35	0.26	0.40	0.31	n.s.	0.30	0.40	0.18	n.s.	0.30	0.25	0.49	n.s.	n.s.	-0.27	n.s.	n.s.	n.s.	0.14	n.s.	-0.24	-0.38	n.s.	0.16
fa44	n.s.	n.s.	n.s.	n.s.	-0.13	n.s.	n.s.	-0.16	n.s.	0.13	n.s.	0.27	n.s.	n.s.	n.s.	n.s.	0.23	n.s.	n.s.	0.13	n.s.	n.s.	n.s.	n.s.
fa45	n.s.	n.s.	-0.18	-0.17	0.17	n.s.	-0.21	n.s.	0.15	-0.24	n.s.	-0.20	n.s.	0.21	n.s.	n.s.	n.s.	n.s.	n.s.	0.13	n.s.	0.16	n.s.	n.s.
fa46	n.s.	0.16	n.s.	n.s.	-0.14	n.s.	-0.14	-0.25	0.19	n.s.	n.s.	n.s.	-0.13	0.20	-0.27	n.s.	-0.30	n.s.	n.s.	0.43	0.25	0.22	n.s.	n.s.
fa48	-0.74	-0.60	-0.85	-0.71	-0.32	-0.65	-0.86	-0.45	-0.24	-0.64	-0.38	-0.47	-0.75	n.s.	0.40	-0.20	n.s.	0.17	0.25	0.19	0.45	0.72	0.23	n.s.
fa49	-0.21	-0.15	-0.19	n.s.	n.s.	n.s.	-0.19	n.s.	-0.13	n.s.	n.s.	n.s.	-0.44	0.26	n.s.	-0.17	n.s.	n.s.	0.46	n.s.	n.s.	n.s.	0.97	0.84
fa50	-0.18	n.s.	n.s.	n.s.	-0.21	n.s.	n.s.	n.s.	-0.23	n.s.	-0.23	0.17	-0.26	0.20	n.s.	-0.19	0.23	n.s.	0.33	n.s.	-0.23	n.s.	0.62	0.63
fa51	-0.20	-0.14	-0.18	n.s.	n.s.	n.s.	-0.16	n.s.	-0.14	n.s.	n.s.	n.s.	-0.40	0.25	n.s.	-0.18	0.14	n.s.	0.46	n.s.	n.s.	n.s.	0.95	0.85

Annex 3 continued (5/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	fa28	fa29	fa30	fa31	fa32	fa33	fa34	fa35	fa36	fa37	fa38	fa39	fa40	fa41	fa42	fa43	fa44	fa45	fa46	fa48	fa49	fa50	fa51	fa52
fa16																								
fa17																								
fa18																								
fa19																								
fa20																								
fa21																								
fa22																								
fa23																								
fa24																								
fa25																								
fa26																								
fa27																								
fa28																								
fa29	0.79																							
fa30	0.21	0.29																						
fa31	0.51	0.62	0.68																					
fa32	-0.31	-0.19	0.27	n.s.																				
fa33	n.s.	n.s.	0.48	0.28	0.58																			
fa34	n.s.	0.13	0.49	0.43	0.50	0.63																		
fa35	n.s.	n.s.	n.s.	0.19	0.28	0.39	0.62																	
fa36	n.s.	0.22	0.46	0.23	0.32	0.26	0.14	-0.39																
fa37	0.17	n.s.	0.39	0.39	n.s.	0.40	0.45	0.34	n.s.															
fa38	0.13	0.19	0.42	0.39	0.24	0.38	0.42	0.33	0.28	0.46														
fa39	n.s.	n.s.	0.20	0.29	0.40	0.50	0.55	0.75	-0.18	0.30	0.30													
fa40	n.s.	n.s.	n.s.	n.s.	n.s.	-0.13	-0.34	-0.17	n.s.	-0.15	-0.15	n.s.												
fa41	n.s.	-0.18	n.s.	n.s.	0.16	0.21	0.16	n.s.	n.s.	n.s.	-0.18	n.s.	0.41											
fa42	0.57	0.61	0.22	0.45	n.s.	0.33	0.15	n.s.	n.s.	0.26	0.18	0.33	0.31	0.21										
fa43	-0.37	-0.33	n.s.	n.s.	0.22	0.19	0.23	0.16	n.s.	0.34	n.s.	n.s.	n.s.	0.27	n.s.									
fa44	n.s.	n.s.	0.17	0.16	n.s.	0.20	0.20	n.s.	n.s.	0.44	n.s.	0.16	n.s.	n.s.	n.s.	0.49								
fa45	n.s.	n.s.	0.34	n.s.	n.s.	n.s.	n.s.	-0.14	0.25	n.s.	0.16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.							
fa46	n.s.	n.s.	0.16	n.s.	n.s.	n.s.	-0.20	-0.45	0.33	n.s.	n.s.	-0.14	0.38	0.17	n.s.	n.s.	0.27	n.s.						
fa48	1.00	0.82	0.24	0.55	-0.29	n.s.	n.s.	n.s.	n.s.	0.18	0.15	n.s.	n.s.	n.s.	0.58	-0.36	n.s.	n.s.	n.s.					
fa49	0.15	0.32	0.55	0.56	0.56	0.72	0.68	0.41	0.40	0.39	0.49	0.55	n.s.	0.14	0.55	n.s.	n.s.	n.s.	n.s.	0.18				
fa50	n.s.	n.s.	0.53	0.46	0.39	0.71	0.73	0.53	0.16	0.89	0.57	0.51	-0.22	n.s.	0.30	0.34	0.38	n.s.	n.s.	0.13	0.67			
fa51	n.s.	0.29	0.56	0.55	0.59	0.73	0.74	0.46	0.40	0.40	0.51	0.57	n.s.	0.15	0.50	n.s.	n.s.	n.s.	n.s.	0.15	0.99	0.70		

Annex 3 continued (6,7/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	EB	DMI	BW	mreq	DIM	NEL	OS	CP	UCP	RNB	CF	CL	MYw	MYs	ECM	Mpr	Mfa	Mla	Mur	SCC	FPR	fa01	fa02	fa03
fa52	0.20	n.s.	-0.14	-0.14	n.s.	n.s.	n.s.	n.s.	n.s.	0.14	0.24	-0.21	-0.18	-0.20	-0.27	n.s.	-0.21	n.s.	0.19	n.s.	-0.23	n.s.	0.38	0.40
fa53	-0.29	-0.26	n.s.	n.s.	n.s.	-0.17	0.28	0.38	0.32	0.24	n.s.	n.s.	n.s.	n.s.	n.s.	-0.13	-0.16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.14
fa54	n.s.	n.s.	0.22	0.22	0.14	0.31	0.17	n.s.	0.26	n.s.	-0.29	0.19	0.18	0.15	n.s.	n.s.	-0.36	n.s.	n.s.	n.s.	-0.33	n.s.	-0.27	-0.19
fa55	0.59	0.42	n.s.	n.s.	0.50	n.s.	n.s.	n.s.	-0.16	0.16	0.15	-0.31	-0.25	-0.27	-0.20	0.41	n.s.	n.s.	0.27	n.s.	n.s.	-0.15	0.49	0.64
fa56	-0.61	-0.43	n.s.	n.s.	-0.54	n.s.	n.s.	n.s.	0.13	-0.16	n.s.	0.29	0.24	0.26	0.20	-0.41	n.s.	n.s.	-0.27	n.s.	0.17	0.17	-0.47	-0.64
fa57	n.s.	n.s.	0.17	0.17	0.14	0.26	0.25	0.21	0.33	n.s.	-0.30	0.22	0.17	0.15	n.s.	n.s.	-0.37	n.s.	n.s.	n.s.	-0.34	n.s.	-0.28	-0.21
fa58	-0.59	-0.42	n.s.	n.s.	-0.50	n.s.	n.s.	n.s.	0.16	-0.16	-0.15	0.31	0.25	0.27	0.20	-0.41	n.s.	n.s.	-0.27	n.s.	n.s.	0.15	-0.49	-0.64
fa59	0.26	0.27	n.s.	n.s.	0.17	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.20	n.s.	n.s.	n.s.	n.s.	n.s.	0.19	0.90	0.97
fa60	0.60	0.50	n.s.	n.s.	0.59	n.s.	n.s.	n.s.	n.s.	0.25	0.15	-0.38	-0.18	-0.21	-0.15	0.43	n.s.	-0.27	0.28	n.s.	-0.22	-0.33	0.21	0.41
fa61	-0.59	-0.50	n.s.	n.s.	-0.57	n.s.	n.s.	n.s.	n.s.	-0.23	-0.14	0.37	0.17	0.20	0.14	-0.43	n.s.	0.24	-0.27	n.s.	0.20	0.27	-0.34	-0.54
fa63	0.33	0.32	0.22	0.22	0.18	0.46	-0.17	-0.33	-0.16	-0.35	-0.28	n.s.	n.s.	n.s.	n.s.	n.s.	-0.13	n.s.	n.s.	n.s.	-0.16	-0.13	n.s.	n.s.
fa65	0.59	0.49	n.s.	n.s.	0.52	n.s.	n.s.	n.s.	n.s.	0.13	n.s.	-0.22	n.s.	-0.15	n.s.	0.41	n.s.	n.s.	0.32	n.s.	-0.33	-0.24	0.54	0.77
fa66	0.42	0.37	n.s.	n.s.	0.45	-0.16	n.s.	n.s.	-0.22	0.27	0.21	-0.42	-0.18	-0.20	n.s.	0.32	0.16	-0.36	0.14	n.s.	n.s.	-0.22	n.s.	0.13
fa67	0.60	0.49	n.s.	n.s.	0.54	n.s.	n.s.	n.s.	n.s.	0.13	n.s.	-0.21	n.s.	-0.16	-0.13	0.42	n.s.	n.s.	0.33	n.s.	-0.35	-0.30	0.51	0.76
fa68	0.60	0.48	n.s.	n.s.	0.55	n.s.	n.s.	n.s.	n.s.	0.21	n.s.	-0.33	-0.17	-0.21	-0.15	0.47	n.s.	-0.18	0.31	n.s.	-0.26	-0.30	0.41	0.64
fa69	0.45	0.20	n.s.	n.s.	0.35	n.s.	n.s.	0.17	n.s.	0.14	n.s.	-0.20	-0.16	-0.20	-0.28	0.30	-0.36	n.s.	0.19	n.s.	-0.52	-0.32	n.s.	0.23
fa78	0.61	0.44	n.s.	n.s.	0.54	0.22	n.s.	n.s.	n.s.	n.s.	-0.14	n.s.	n.s.	-0.15	-0.17	0.47	-0.20	-0.13	0.20	n.s.	-0.45	-0.32	n.s.	0.23

Abbr	fa04	fa05	fa06	fa07	fa08	fa09	fa10	fa11	fa12	fa13	fa14	fa15	fa16	fa17	fa18	fa19	fa20	fa21	fa22	fa23	fa24	fa25	fa26	fa27
fa52	0.35	0.33	0.24	n.s.	n.s.	0.17	0.22	n.s.	0.50	n.s.	0.58	0.60	-0.14	0.22	-0.51	0.42	-0.16	n.s.	0.46	0.66	n.s.	0.27	n.s.	n.s.
fa53	-0.16	-0.15	-0.20	-0.22	n.s.	-0.13	-0.22	n.s.	n.s.	-0.14	n.s.	0.14	-0.32	0.28	n.s.	n.s.	0.17	n.s.	0.41	n.s.	n.s.	0.31	0.42	0.52
fa54	-0.15	n.s.	n.s.	n.s.	-0.21	n.s.	n.s.	n.s.	-0.25	n.s.	-0.24	0.19	-0.25	0.14	n.s.	-0.20	0.25	n.s.	0.30	n.s.	-0.20	n.s.	0.52	0.59
fa55	0.75	0.56	0.82	0.60	0.35	0.55	0.83	0.30	0.30	0.54	0.42	0.41	0.75	-0.14	-0.44	0.28	n.s.	-0.18	-0.34	-0.13	-0.31	-0.65	-0.47	-0.26
fa56	-0.76	-0.57	-0.84	-0.63	-0.34	-0.57	-0.85	-0.33	-0.29	-0.57	-0.42	-0.45	-0.75	n.s.	0.45	-0.27	n.s.	0.18	0.31	0.15	0.35	0.67	0.41	0.19
fa57	-0.17	n.s.	-0.13	n.s.	-0.20	n.s.	n.s.	n.s.	-0.17	n.s.	-0.18	0.20	-0.28	0.22	n.s.	-0.16	0.24	n.s.	0.37	n.s.	-0.19	n.s.	0.61	0.65
fa58	-0.75	-0.56	-0.82	-0.60	-0.35	-0.55	-0.83	-0.30	-0.30	-0.54	-0.42	-0.41	-0.75	0.14	0.44	-0.28	n.s.	0.18	0.34	0.13	0.31	0.65	0.47	0.26
fa59	0.91	0.68	0.77	0.43	n.s.	0.47	0.60	n.s.	0.25	0.27	0.29	0.27	0.23	n.s.	-0.52	0.15	-0.36	-0.16	-0.25	0.28	-0.15	-0.34	-0.30	n.s.
fa60	0.58	0.58	0.75	0.72	0.23	0.69	0.81	0.60	n.s.	0.67	0.24	0.40	0.92	n.s.	n.s.	n.s.	n.s.	n.s.	-0.34	-0.24	-0.71	-0.70	-0.38	-0.24
fa61	-0.69	-0.64	-0.82	-0.73	-0.22	-0.71	-0.84	-0.57	-0.15	-0.65	-0.27	-0.41	-0.88	n.s.	0.16	n.s.	n.s.	0.14	0.36	0.18	0.68	0.70	0.40	0.23
fa63	n.s.	n.s.	n.s.	0.29	n.s.	n.s.	0.16	0.16	-0.32	0.24	-0.24	n.s.	0.14	-0.20	n.s.	-0.24	n.s.	n.s.	-0.17	-0.13	-0.26	-0.37	n.s.	n.s.
fa65	0.90	0.69	0.97	0.76	0.29	0.72	0.95	0.47	0.24	0.63	0.46	0.56	0.52	n.s.	-0.47	0.20	n.s.	-0.14	-0.14	n.s.	-0.49	-0.64	-0.24	n.s.
fa66	0.26	0.40	0.41	0.48	n.s.	0.49	0.48	0.50	n.s.	0.49	n.s.	0.13	0.99	n.s.	0.21	n.s.	n.s.	n.s.	-0.47	-0.24	-0.67	-0.55	-0.44	-0.35
fa67	0.90	0.68	0.97	0.78	0.28	0.73	0.96	0.48	0.23	0.64	0.47	0.56	0.52	n.s.	-0.46	0.20	n.s.	-0.14	-0.13	n.s.	-0.50	-0.64	-0.23	n.s.
fa68	0.80	0.68	0.91	0.79	0.22	0.74	0.90	0.54	0.18	0.69	0.34	0.48	0.74	n.s.	-0.27	0.14	n.s.	-0.15	-0.27	n.s.	-0.63	-0.65	-0.34	-0.20
fa69	0.39	0.37	0.47	0.72	n.s.	0.36	0.52	0.28	n.s.	0.88	0.34	0.77	0.29	n.s.	-0.24	n.s.	0.47	n.s.	0.28	n.s.	-0.48	-0.22	n.s.	0.18
fa78	0.45	0.38	0.63	0.81	0.33	0.48	0.74	0.42	n.s.	0.85	0.31	0.53	0.58	-0.21	-0.18	n.s.	0.24	n.s.	-0.16	-0.48	-0.50	-0.68	n.s.	n.s.

Annex 3 continued (8,9/9) Correlations with |R|≥0.125 are significant at the 5% level; n.s. = non-significant. (see Annex 1 for the abbreviation code (Abbr))

Abbr	fa28	fa29	fa30	fa31	fa32	fa33	fa34	fa35	fa36	fa37	fa38	fa39	fa40	fa41	fa42	fa43	fa44	fa45	fa46	fa48	fa49	fa50	fa51	fa52
fa52	n.s.	n.s.	n.s.	-0.16	n.s.	n.s.	n.s.	-0.35	0.38	n.s.	0.18	-0.38	0.17	0.31	n.s.	0.27	0.15	n.s.	0.31	n.s.	n.s.	n.s.	n.s.	
fa53	0.16	0.20	0.45	0.40	0.25	0.40	0.40	0.30	0.32	0.42	0.96	0.33	n.s.	-0.14	0.21	n.s.	0.21	0.38	n.s.	0.18	0.49	0.54	0.51	0.20
fa54	n.s.	n.s.	0.49	0.45	0.31	0.57	0.69	0.52	n.s.	0.94	0.55	0.47	-0.21	n.s.	0.25	0.41	0.46	n.s.	-0.14	n.s.	0.58	0.98	0.61	n.s.
fa55	-0.94	-0.82	-0.39	-0.65	n.s.	-0.32	-0.27	n.s.	-0.19	-0.35	-0.30	-0.27	n.s.	n.s.	-0.66	0.28	n.s.	n.s.	n.s.	-0.95	-0.44	-0.38	-0.42	0.15
fa56	0.96	0.84	0.34	0.61	-0.15	0.24	0.19	n.s.	0.17	0.25	0.22	0.22	n.s.	n.s.	0.65	-0.34	n.s.	n.s.	n.s.	0.97	0.37	0.27	0.35	-0.16
fa57	n.s.	0.13	0.55	0.47	0.39	0.70	0.72	0.52	0.20	0.87	0.67	0.51	-0.20	n.s.	0.29	0.37	0.43	n.s.	n.s.	0.13	0.67	0.99	0.70	n.s.
fa58	0.94	0.82	0.39	0.65	n.s.	0.32	0.27	n.s.	0.19	0.35	0.30	0.27	n.s.	n.s.	0.66	-0.28	n.s.	n.s.	n.s.	0.95	0.44	0.38	0.42	-0.15
fa59	-0.63	-0.49	n.s.	-0.33	n.s.	-0.23	-0.21	-0.32	0.13	-0.18	n.s.	-0.50	n.s.	n.s.	-0.43	0.26	n.s.	n.s.	n.s.	-0.63	-0.24	-0.24	-0.25	0.36
fa60	-0.89	-0.74	-0.36	-0.65	0.20	n.s.	n.s.	n.s.	-0.21	-0.23	-0.26	n.s.	-0.34	n.s.	-0.66	0.27	n.s.	-0.15	-0.17	-0.90	-0.36	-0.19	-0.32	n.s.
fa61	0.92	0.76	0.34	0.66	-0.18	0.15	n.s.	n.s.	0.17	0.24	0.26	0.20	0.29	n.s.	0.68	-0.30	n.s.	0.14	0.13	0.93	0.37	0.22	0.34	n.s.
fa63	n.s.	-0.15	n.s.	n.s.	n.s.	n.s.	0.17	0.14	-0.25	0.39	-0.52	n.s.	n.s.	0.19	n.s.	0.21	0.14	-0.43	-0.21	n.s.	n.s.	0.29	n.s.	-0.24
fa65	-0.88	-0.70	-0.26	-0.52	0.21	n.s.	n.s.	n.s.	n.s.	-0.16	-0.15	-0.21	n.s.	0.17	-0.50	0.41	n.s.	-0.17	n.s.	-0.89	-0.20	n.s.	-0.17	0.29
fa66	-0.68	-0.59	-0.32	-0.60	n.s.	-0.16	n.s.	n.s.	-0.20	-0.26	-0.29	n.s.	-0.42	-0.19	-0.66	n.s.	n.s.	n.s.	-0.16	-0.69	-0.44	-0.25	-0.40	-0.20
fa67	-0.88	-0.69	-0.27	-0.53	0.23	n.s.	n.s.	n.s.	n.s.	-0.15	-0.15	-0.21	n.s.	0.18	-0.49	0.41	n.s.	-0.18	n.s.	-0.88	-0.19	n.s.	-0.16	0.29
fa68	-0.91	-0.72	-0.35	-0.63	0.20	-0.13	n.s.	n.s.	-0.13	-0.23	-0.22	-0.22	-0.23	n.s.	-0.60	0.33	n.s.	-0.17	n.s.	-0.92	-0.32	-0.20	-0.29	0.17
fa69	-0.50	-0.22	-0.17	-0.21	0.25	0.13	0.36	0.20	n.s.	n.s.	n.s.	n.s.	-0.15	0.19	-0.17	0.37	0.26	-0.17	n.s.	-0.49	n.s.	0.18	0.14	0.46
fa78	-0.75	-0.54	-0.24	-0.36	0.20	n.s.	0.25	0.34	-0.28	n.s.	-0.15	0.17	-0.16	n.s.	-0.37	0.32	n.s.	-0.27	-0.16	-0.75	n.s.	n.s.	n.s.	n.s.

Abbr	fa53	fa54	fa55	fa56	fa57	fa58	fa59	fa60	fa61	fa63	fa65	fa66	fa67	fa68	fa69
fa52															
fa53															
fa54	0.52														
fa55	-0.32	-0.34													
fa56	0.25	0.23	-0.99												
fa57	0.66	0.97	-0.38	0.27											
fa58	0.32	0.34	-1.00	0.99	0.38										
fa59	-0.13	-0.20	0.66	-0.66	-0.22	-0.66									
fa60	-0.31	-0.18	0.87	-0.87	-0.21	-0.87	0.43								
fa61	0.30	0.20	-0.91	0.91	0.23	0.91	-0.56	-0.99							
fa63	-0.61	0.33	n.s.	n.s.	0.15	n.s.	n.s.	0.18	-0.16						
fa65	-0.20	n.s.	0.85	-0.87	-0.13	-0.85	0.78	0.79	-0.86	n.s.					
fa66	-0.31	-0.24	0.69	-0.68	-0.27	-0.69	0.16	0.89	-0.85	0.15	0.45				
fa67	-0.20	n.s.	0.85	-0.87	n.s.	-0.85	0.75	0.80	-0.86	0.13	1.00	0.46			
fa68	-0.27	-0.18	0.89	-0.90	-0.21	-0.89	0.65	0.93	-0.96	0.13	0.94	0.70	0.94		
fa69	n.s.	0.20	0.39	-0.43	0.19	-0.39	0.23	0.47	-0.47	n.s.	0.55	0.26	0.56	0.55	
fa78	-0.20	n.s.	0.66	-0.69	n.s.	-0.66	0.26	0.75	-0.73	0.24	0.70	0.55	0.70	0.73	0.63

Annex 4 Pearson correlation of total energy balance (EB, n = 248) and negative (n = 137) and positive EB data (n = 111) separately with milk fatty acids (FA in g/100 g FA). Correlations greater than 0.125 in absolute value, i.e. with |r|≥0.125, are significant at the 5%-level

	EB				EB		
milk FA	total	negative	positive	milk FA	total	negative	positive
C4:0	-0.30	-0.18	n.s.	C18:2t11,c15+t9,c12	-0.15	n.s.	n.s.
C6:0	n.s.	0.24	-0.23	C18:2c9,c12	n.s.	n.s.	n.s.
C8:0	0.27	0.37	-0.20	C18:3c9,c12,c15	-0.28	n.s.	n.s.
C10:0	0.41	0.45	n.s.	C19:0	n.s.	n.s.	n.s.
C10:1	0.35	0.45	n.s.	C20:0	n.s.	0.19	n.s.
C12:0	0.55	0.49	n.s.	C20:1c9	0.27	0.37	n.s.
C12:1*cis*+C13:0	0.54	0.52	n.s.	C20:1c11	-0.38	n.s.	n.s.
C13:0*iso*	0.33	0.23	n.s.	C20:3*n-6*	0.39	0.43	n.s.
C13:0*anteiso*	0.47	0.46	n.s.	C20:4*n-6*	n.s.	n.s.	n.s.
C14:0	0.62	0.53	0.21	C20:5*n-3*	-0.23	n.s.	n.s.
C14:1*cis*	0.38	0.28	0.23	C22:0	n.s.	n.s.	n.s.
C14:0*iso*	0.28	0.22	0.20	C22:5*cis*	n.s.	n.s.	n.s.
C15:0	0.51	0.50	n.s.	∑C18:1*cis*	-0.63	-0.55	n.s.
C15:0*iso*	0.45	0.41	0.28	∑C18:1*trans*	-0.13	n.s.	n.s.
C15:0*anteiso*	0.46	0.47	n.s.	∑C18:2	n.s.	n.s.	n.s.
C16:0	0.46	0.30	n.s.	∑*trans*	n.s.	n.s.	n.s.
C16:1*trans*	n.s.	n.s.	n.s.	BCFA	0.20	0.42	n.s.
C16:1*cis*	-0.28	-0.41	n.s.	∑*n-3*	-0.29	n.s.	n.s.
C16:0*iso*	0.25	0.25	0.21	∑*n-6*	n.s.	n.s.	n.s.
C17:0	n.s.	n.s.	n.s.	SFA	0.59	0.51	n.s.
C17:1	n.s.	n.s.	n.s.	MUFA	-0.61	-0.53	n.s.
C17:0*iso*	n.s.	n.s.	n.s.	PUFA	n.s.	n.s.	n.s.
C17:0*anteiso*	-0.17	n.s.	-0.24	UFA	-0.59	-0.51	n.s.
C18:0	-0.33	-0.17	-0.19	SCFA	0.26	0.36	n.s.
C18:0*iso*	-0.55	-0.35	n.s.	MCFA	0.60	0.46	n.s.
C18:1t6-t11	-0.13	n.s.	n.s.	LCFA	-0.59	-0.47	n.s.
C18:1t12-t14	n.s.	n.s.	n.s.	*n-6/n-3*	0.33	0.17	n.s.
C18:1c9	-0.62	-0.55	n.s.	∑C4-15	0.59	0.53	n.s.
C18:1c11	-0.55	-0.42	n.s.	∑C16	0.42	0.24	n.s.
C18:1c12	-0.26	n.s.	n.s.	∑C6-15	0.60	0.54	n.s.
C18:1c13+c14+t16	-0.40	-0.28	n.s.	C6-15/LCFA	0.60	0.50	n.s.
C18:2t,t-NMID	0.22	0.20	0.25	OCFA	0.45	0.50	n.s.
C18:2c9,t11	n.s.	n.s.	0.28	C15/C17	0.61	0.47	0.29
C18:2c9,t13+t8,c12	n.s.	n.s.	n.s.	OA/*de novo*	-0.62	-0.54	n.s.
C18:2c9,t12+t8,c13	n.s.	n.s.	0.25				

FA = fatty acid; c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA; SFA = saturated FA; UFA = unsaturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SCFA = short-chain FA (<C12); MCFA = medium-chain FA (C12-C16); LCFA = long-chain FA(>C16); OCFA = odd-chain FA; BCFA = branched-chain FA; OA/*de novo* = C18:1c9/(C6:0+C8:0+C10:0+C12:0+C14:0)

Annex 5 Number of models from five-fold cross-validation of the regularized linear regression models which the respective effects entered and the 30 most important variables from predicting energy balance with random forests (FA in g/100 g FA)

Effect	Lasso	ENET	Ada Lasso	ADA ENET	Random Forests	Effect	Lasso	ENET	Ada Lasso	ADA ENET	Random Forests
Parity	-	-	-	-		C16:0*iso*	4	4	1	-	x
DIM (d)	5	5	4	2	x	C17:0	2	1	1	-	
NEL (MJ/kg DM)	3	2	-	-		C17:1	-	-	-	-	
OM (g/kg DM)	1	1	-	-	x	C17:0*iso*	-	-	-	-	
CP (g/kg DM)	-	-	-	-	x	C17:0*anteiso*	-	-	-	-	
uCP (g/kg DM)	2	2	1	-		C18:0	-	-	-	-	
RNB (g/kg DM)	4	1	2	-	x	C18:0*iso*	5	5	5	5	x
CF (g/kg DM)	4	2	1	-		C18:1t6-t11	-	-	-	-	x
CL (g/kg DM)	1	-	-	-		C18:1t12-t14	1	1	-	-	
MYs	5	5	5	5	x	C18:1c9	5	5	4	4	x
Milk protein (%)	3	3	1	-		C18:1c11	1	1	1	1	x
Milk fat (%)	5	5	5	3		C18:1c12	-	-	-	-	
Milk lactose (%)	-	-	-	-		C18:1c13+c14+t16	-	-	-	-	
C4:0	2	2	-	-		C18:2t,t-NMID	-	-	-	-	
C6:0	-	1	1	-		C18:2c9,t11	3	2	2	-	x
C8:0	2	1	-	-		C18:2c9,t13+t8,c12	-	-	-	-	
C10:0	-	-	-	-	x	C18:2c9,t12+t8,c13	3	1	-	-	
C10:1	-	-	-	-		C18:2t11,c15+t9,c12	-	-	-	-	
C12:0	-	-	-	-	x	C18:2c9,c12	-	-	1	-	
C12:1*cis*+C13:0	5	5	3	3	x	C18:3c9,c12,c15	3	4	2	-	x
C13:0*iso*	2	1	-	-	x	C19:0	2	1	1	-	
C13:0*anteiso*	-	1	-	1	x	C20:0	2	1	2	-	
C14:0	1	3	-	1	x	C20:1c9	-	-	-	-	
C14:1*cis*	1	1	-	-	x	C20:1c11	4	4	2	-	x
C14:0*iso*	5	5	3	1	x	C20:3*n-6*	5	5	-	1	x
C15:0	-	1	-	-	x	C20:4*n-6*	1	-	-	-	
C15:0*iso*	5	5	5	5	x	C20:5*n-3*	4	2	-	-	x
C15:0*anteiso*	-	-	-	-	x	C22:0	2	1	2	-	
C16:0	2	3	1	-	x	*n-6/n-3*	5	5	5	4	x
C16:1*trans*	1	1	-	-		C6-C15/LCFA	-	1	-	1	x
C16:1*cis*	2	1	1	-		C15/C17	4	5	4	5	x

FA = fatty acid; ENET = eleastic net; AdaLasso = adaptive lasso; ADAENET = adaptive elastic net; DIM = days in milk; NEL = net energy for lactation; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; RNB = ruminal nitrogen balance CF = crude fiber; CL = crude fat; MYs = milk yield at day of sampling; c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA

Annex 6 Accuracy of energy balance predictions of the regularized linear regression models and random forests for each single validation subset from 5-fold cross-validation

Ridge Regression			Lasso			Elastic net		
Subset	RMSE	r	Subset	RMSE	r	Subset	RMSE	r
A	15.60	0.79	A	15.07	0.80	A	15.85	0.80
B	15.63	0.74	B	15.96	0.73	B	15.42	0.75
C	14.08	0.87	C	14.94	0.85	C	14.96	0.85
D	13.26	0.84	D	13.08	0.85	D	13.09	0.85
E	16.45	0.83	E	16.19	0.84	E	16.27	0.84
TOTAL	15.05	0.81	TOTAL	15.09	0.81	TOTAL	15.16	0.82

Adaptive lasso			Adaptive elastic net				Random forests		
Subset	RMSE	r	Subset	opt. gamma	RMSE	r		RMSE	r
A	15.25	0.81	A	2.00	19.89	0.79			
B	15.69	0.75	B	0.10	15.83	0.74			
C	15.31	0.84	C	0.20	15.46	0.84			
D	13.21	0.84	D	0.10	17.68	0.83			
E	16.06	0.84	E	1.00	16.61	0.82			
TOTAL	15.14	0.81	TOTAL		17.17	0.80	TOTAL	16.02	0.80

RMSE = root mean square error of prediction; r = Pearson correlation coefficient of predicted with observed energy balance data

Annex 7 Estimates of the coefficients of the full model for prediction of energy balance (FA in g/100g FA; bold printed estimates are significant at the 5%-level)

Effect	Estimate	Effect	Estimate	Effect	Estimate
Intercept	**19217.7**	C13:0*iso*	-246.7	C18:1c11	-162.2
Parity = 1	-2.6	C13:0*anteiso*	**-275.4**	C18:1c12	**-192.2**
Parity > 1	0.0	C14:0	**-197.8**	C18:1c13+c14+t16	**-200.5**
DIM (d)	**0.1**	C14:1*cis*	**-191.7**	C18:2t,t-NMID	**-317.6**
NEL (MJ/kg DM)	13.4	C14:0*iso*	-43.1	C18:2c9,t11	-174.0
OM (g/kg DM)	0.6	C15:0	**-238.9**	C18:2c9,t13+t8,c12	-121.2
CP (g/kg DM)	7.1	C15:0*iso*	-93.5	C18:2c9,t12+t8,c13	**-227.4**
uCP (g/kg DM)	-7.4	C15:0*anteiso*	**-210.3**	C18:2t11,c15+t9,c12	**-221.4**
RNB (g/kg DM)	-40.9	C16:0	**-198.2**	C18:2c9,c12	-192.9
CF (g/kg DM)	0.1	C16:1*trans*	-95.2	C18:3c9,c12,c15	**-187.7**
CL (g/kg DM)	0.0	C16:1*cis*	**-197.8**	C19:0	**-252.0**
MYs	**-1.1**	C16:0*iso*	**-186.1**	C20:0	-61.5
Milk protein (%)	4.4	C17:0	**-231.5**	C20:1c9	**-244.4**
Milk fat (%)	**-8.3**	C17:1	-216.9	C20:1c11	**-385.3**
Milk lactose (%)	2.9	C17:0*iso*	-95.4	C20:3*n-6*	-144.8
C4:0	**-197.4**	C17:0*anteiso*	**-205.9**	C20:4*n-6*	**-254.0**
C6:0	**-192.5**	C18:0	**-197.3**	C20:5*n-3*	**-207.1**
C8:0	**-202.2**	C18:0*iso*	**-317.8**	C22:0	**-296.3**
C10:0	**-202.3**	C18:1t6-t11	**-200.8**	*n-6/n-3*	9.3
C10:1	**-230.9**	C18:1t12-t14	**-212.5**	C6-C15/LCFA	33.8
C12:0	**-210.7**	C18:1c9	**-201.7**	C15/C17	5.3
C12:1*cis*+C13:0	102.9				

FA = fatty acid; DIM = days in milk; NEL = net energy for lactation; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; RNB = ruminal nitrogen balance CF = crude fiber; CL = crude fat; MYs = milk yield at day of sampling; c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA; SFA = saturated FA; UFA = unsaturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SCFA = short-chain FA (<C12); MCFA = medium-chain FA (C12-C16); LCFA = long-chain FA(>C16); OCFA = odd-chain FA; BCFA = branched-chain FA

Annex 8 Estimates of the coefficients of all models for prediction of energy balance obtained after variable selection with Lasso, Elastic net, and Adaptive lasso (FA in g/100g FA; bold printed estimates are significant at the 5%-level)

	Lasso MODEL			Eleastic net MODEL			Adaptive lasso MODEL			
Effect	1	2	3	1	2	3	1	2	3	4
Intercept	**-309.2**	-3.4	23.5	**-397.9**	26.9	**68.8**	-34.5	-38.8	-22.3	10.0
DIM (d)	**0.1**	**0.1**	**0.1**	0.1	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**
NEL (MJ/kg DM)	**16.4**			17.5						
OM (g/kg DM)	**0.2**			0.3						
uCP (g/kg DM)	**-0.1**			-0.2			0.0			
RNB (g/kg DM)	**2.3**			**2.3**			**2.1**			**2.1**
CF (g/kg DM)	**0.1**			0.1			0.0	**0.2**	**0.1**	
CL (g/kg DM)	**0.3**									
MYs (kg)	**-1.1**	**-1.2**	**-1.2**	**-1.1**	**-1.1**	**-1.2**	**-1.0**	**-1.2**	**-1.2**	**-1.1**
Milk protein (%)	3.4			2.6			4.2			
Milk fat (%)	**-9.3**	**-6.2**	**-6.7**	**-10.1**	**-7.2**	**-8.2**	**-7.7**	**-7.3**	**-7.8**	**-7.7**
C4:0	**3.6**			2.8						
C6:0				17.1			-13.6			**-16.2**
C8:0	**-27.4**	-12.1	**-13.3**	-44.7	**-21.7**	**-23.4**				
C12:1*cis*+C13:0	**108.9**			**219.9**			62.2			**66.6**
C13:0*iso*	46.9			-42.9						
C13:0*anteiso*				-65.5						
C14:0	0.5	0.4		2.2	0.7					
C14:1*cis*	-1.7			1.7						
C14:0*iso*	**133.7**	**112.5**		91.0	**103.0**		**156.2**	105.2		**148.6**
C15:0				-24.7						
C15:0*iso*	131.5	**164.6**	**223.1**	**138.3**	**136.5**	**186.1**	**172.1**	**129.9**	**159.9**	**195.3**
C16:0	0.1			0.2			0.5			
C16:1*trans*	1.0			33.2						
C16:1*cis*	**5.5**			6.5			5.6			**7.2**
C16:0*iso*	16.6			21.0			17.8			
C17:0	**-26.7**			-7.1			-21.0	**-18.9**	**-21.5**	**-33.3**
C18:0*iso*	**-192.6**			-160.3			-181.2			
C18:1t12-t14	-7.3			-8.7						
C18:1c9	**-1.8**	**-2.3**	**-2.7**	**-2.5**	**-2.4**	**-2.9**	-1.5	**-1.7**	**-1.4**	**-2.2**
C18:1c11	**20.0**	**16.4**	**15.0**	**18.7**	7.2		**26.4**	7.9		**23.2**
C18:2c9,t11	**27.1**			25.5			**24.5**			
C18:2c9,t12+t8,c13	2.6			-14.2	-2.8					
C18:2c9,c12							**14.1**			**14.5**
C18:3c9,c12,c15	4.6			11.2			-43.3			**-45.2**
C19:0	-35.2			-52.1	**-59.9**	**-59.4**	-37.1			
C20:0	**128.1**			**125.4**			**146.1**			**133.4**
C20:1c11	**-179.9**	**-126.0**	**-121.5**	**-159.1**			**-190.6**			**-205.6**
C20:3*n-6*	53.7	51.0		65.4	49.1					
C20:4*n-6*	2.3									
C20:5*n-3*	-48.1			-50.6						
C22:0	**-112.1**			-91.9			**-118.0**			**-113.1**
n-6/n-3	**7.0**	**9.9**	**10.0**	**6.9**	**9.1**	**9.1**	**5.1**	**9.8**	**9.0**	**5.7**
C6-C15/LCFA				-46.0						
C15/C17	-12.6	5.8		0.0	8.3		-0.9	**17.9**	**18.1**	

DIM = days in milk; NEL = net energy for lactation; OM = organic matter; CP = crude protein; uCP = utilizable crude protein at the duodenum; RNB = ruminal N-balance; CF = crude fiber; CL = crude fat; MYs = milk yield at day of sampling; c = *cis*; t = *trans*; t,t-NMID = *trans*, *trans*-nonmethylene interrupted diene; *n-3* = *omega-3 FA*; *n-6* = *omega-6* FA; LCFA = long chain FA (>C16)

Annex 9 Estimates of the coefficients of all models for prediction of energy balance obtained after variable selection with the Adaptive elastic net, Random forests, stepwise selection (GLMs, no interactions) and of the pre-specified Model MODELpre (FA in g/100g FA; bold printed estimates are significant at the 5%-level)

	Adaptive elastic net MODEL			Random forests MODEL			GLMs		MODEL
Effect	1	2	3	1	2	3	N	FA-N	pre
Intercept	-5.0	-12.2	**23.0**	-160.0	**-66.1**	**-75.9**	15.9	**-129.7**	**82.9**
DIM (d)	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**		
OM (g/kg DM)				0.2					
CP (g/kg DM)				-0.1					
RNB (g/kg DM)				1.0			**2.5**		
MYs	**-1.2**	**-1.2**	**-1.2**	**-1.1**	**-1.1**	**-1.0**	**-1.1**		**-1.4**
Milk fat (%)	**-6.7**	**-6.4**	**-7.6**				**-7.3**		
C6:0								**-21.2**	
C8:0							**-27.1**		
C10:0				-8.3	**-5.8**				
C12:0				-5.8					
C12:1*cis*+C13:0	19.2			**157.8**			**128.8**	**126.9**	
C13:0*iso*				-101.2					
C13:0*anteiso*	0.0			20.4					
C14:0	-1.8	-0.1		2.3	1.8				
C14:1*cis*				-4.6					
C14:0*iso*	**170.3**	**110.6**		**127.5**	**121.9**	**123.4**	**140.4**	**214.5**	
C15:0				-14.3			-13.0	**-20.6**	
C15:0*iso*	**156.5**	**137.6**	**176.1**	**180.0**	**183.5**	**195.8**	**211.3**	**321.2**	
C15:0*anteiso*				10.3					
C16:0				-0.5				**2.1**	
C16:1*cis*							5.8		
C16:0*iso*				**46.7**					
C17:0							**-29.2**		
C18:0*iso*	**-270.7**			**-246.0**					
C18:1t6-t11				-1.8					
C18:1c9	-1.3	**-2.1**	**-2.2**	**-2.6**	**-2.1**	**-1.8**	**-2.7**	**-2.1**	
C18:1c11	13.3	8.0		**26.4**	**21.8**	**20.9**	**27.4**	**31.0**	
C18:2t,t-NMID							-107.6		
C18:2c9,t11				20.2			**24.3**		
C18:2c9,t13+t8,c12								**86.0**	
C18:2c9,c12							**13.7**	**27.8**	
C18:3c9,c12,c15				-19.2			-42.7	**-103.7**	
C20:0							**143.4**	**128.6**	
C20:1c11				**-124.5**	**-120.2**	**-110.6**	**-235.0**	**-211.1**	
C20:3*n-6*	21.3	26.0		67.1	70.0				
C20:5*n-3*				-31.4				**-102.3**	
C22:0							**-115.3**		
n-6/n-3	**8.7**	**9.4**	**9.3**	**7.8**	**10.3**	**10.8**	**6.3**		**7.0**
C6-C15/LCFA	21.3			-7.1					
C15/C17	3.5	10.8		-7.2	9.4	**14.3**			
OA/*de novo*									**-80.5**
FPR									**-34.8**

DIM = days in milk; OM = organic matter; CP = crude protein; RNB = ruminal N-balance; CF = crude fiber; CL = crude fat; MYs = milk yield at day of sampling; FA = fatty acid; c = *cis*; t = *trans*; t,t-NMID = *trans, trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA; LCFA = long chain FA (>C16); OA/*de novo* = C18:1c9/(C6:0+C8:0+C10:0+C12:0+C14:0); FPR = milk fat-to-protein ratio

Annex 10 Coefficients of the models obtained with stepwise selection including interactions with all variables (GLMs-H) and with FA only (GLMs-FA-H, FA in g/100 g FA; bold printed estimates are significant at the 5%-level)

GLMs-H

Effect	Estimate
Intercept	**3153.5**
DIM (d)	**-26.2**
NEL (MJ/kg DM)	**342.1**
DIM*NEL (MJ/kg DM)	-0.3
OM (g/kg DM)	**-5.3**
DIM*OM (g/kg DM)	**0.0**
uCP (g/kg DM)	**-1.6**
CF (g/kg DM)	**10.2**
NEL (MJ/kg DM)*CF (g/kg DM)	**-1.5**
C8:0	**-3347.2**
OM (g/kg DM)*C8:0	**3.5**
C12:1*cis*+C13:0	**5485.7**
DIM*C12:1*cis*+C13:0	**2.5**
OM (g/kg DM)*C12:1*cis*+C13:0	**-6.0**
C14:0*iso*	**3526.1**
uCP (g/kg DM)*C14:0*iso*	**-19.9**
C15:0	**-411.4**
NEL (MJ/kg DM)*C15:0	**60.1**
C15:0*iso*	**-2459.1**
uCP (g/kg DM)*C15:0*iso*	**12.7**
C16:1*cis*	**-53.8**
DIM*C16:1*cis*	**0.3**
C17:1	**-8342.6**
CF (g/kg DM)*C17:1	**38.8**
C8:0*C17:1	**1787.1**
C18:0*iso*	**-1463.8**
CF (g/kg DM)*C18:0*iso*	**11.9**
C8:0*C18:0*iso*	**-461.7**
C18:1c9	**-13.4**
DIM*C18:1c9	**0.0**
C15:0*iso**C18:1c9	**37.9**
C18:1c11	-12.8
C16:1*cis**C18:1c11	**19.7**
C18:2t,t-NMID	**-845.9**
CF (g/kg DM)*C18:2t,t-NMID	**8.8**
C8:0*C18:2t,t-NMID	-381.5
C18:2c9,t11	**170.2**
C16:1*cis**C18:2c9,t11	**-74.2**
C20:0	-97.1
DIM*C20:0	**2.2**
C20:1c11	**-18445.4**
DIM*C20:1c11	**-3.5**
OM (g/kg DM)*C20:1c11	**24.4**
CF (g/kg DM)*C20:1c11	**-15.7**
C15:0*iso**C20:1c11	**-4093.9**
C22:0	**8692.0**
OM (g/kg DM)*C22:0	**-9.4**
C14:0*iso**C22:0	**-5534.6**
n-6/n-3	**111.4**
NEL (MJ/kg DM)**n-6/n-3*	**-17.3**
C15:0*iso***n-6/n-3*	30.0
Milk fat (%)	**-6.3**
MYs	-2.0
uCP (g/kg DM)*MYs	0.0
C8:0*MYs	**2.4**
C15:0*MYs	**-0.8**
C16:1*cis**MYs	**1.0**
C18:2t,t-NMID*MYs	**-12.6**
C20:1c11*MYs	**-18.9**
C22:0*MYs	**10.5**
*n-6/n-3**MYs	**0.2**

GLMs-FA-H

Effect	Estimate
Intercept	-86.7
C6:0	6.1
C12:1*cis*+C13:0	**-360.7**
C14:0*iso*	**1176.1**
C6:0*C14:0*iso*	**-762.0**
C12:1*cis*+C13:0*C14:0*iso*	**2470.2**
C15:0	**-39.4**
C15:0*iso*	192.4
C16:0	**6.4**
C18:1c9	**-11.0**
C15:0*iso**C18:1c9	**21.9**
C18:1c11	**71.2**
C18:2c9,t13+t8,c12	**-474.7**
C12:1*cis*+C13:0*C18:2c9,t13+t8,c12	**1400.0**
C18:1c9*C18:2c9,t13+t8,c12	**25.7**
C18:1c11*C18:2c9,t13+t8,c12	-136.7
C18:2c9,c12	2.7
C18:3c9,c12,c15	**-309.6**
C18:2c9,c12*C18:3c9,c12,c15	**114.5**
C20:0	**771.2**
C16:0*C20:0	**-19.3**
C20:1c11	**525.4**
C15:0*iso**C20:1c11	**-4773.6**
C20:3*n-6*	-364.8
C6:0*C20:3*n-6*	**446.1**
C18:2c9,c12*C20:3*n-6*	**-235.3**
C20:5n-3	226.8
C18:2c9,t13+t8,c12*C20:5*n-3*	**-2636.9**
C20:3*n-6**C20:5*n-3*	3553.6
C22:0	-177.3
C14:0*iso**C22:0	-3429.6
C15:0*C22:0	261.7

FA = fatty acid; DIM = days in milk; NEL = net energy for lactation; OM = organic matter; uCP = utilizable crude protein at the duodenum; CF = crude fiber; MYs = milk yield at day of sampling; MYw = milk yield. weeks mean; c = *cis*; t = *trans*; t.t-NMID = *trans. trans*-nonmethylene interrupted diene; *n-3* = *omega-3* FA; *n-6* = *omega-6* FA;

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PERSONAL DATA

Name:	Vera Becher
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Since 01/2011	Research for PhD, Institute of Animal Science, Animal Nutrition, University of Hohenheim, Stuttgart, Germany
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10/2005 – 09/2008	Undergraduate studies of Agricultural Science with specialization in Animal Science, University of Hohenheim, Stuttgart, Germany Qualification gained: Bachelor of Science
09/1994 – 06/2003	Gymnasium Immenstadt, Immenstadt im Allgäu, Germany Qualification gained: Abitur

PROFESSIONAL CAREER

Since 07/2015	Employee for Research and Development at the SaluVet GmbH, Bad Waldsee, Germany
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PRACTICAL EXPERIENCE

02/2010 – 03/2010	Internship at the Institute of Animal Nutrition of the Bavarian Research Centre for Agriculture, Grub, Germany
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